

AD \_\_\_\_\_

Award Number: DAMD17-02-P-0699

TITLE: Fourth International Meeting on Molecular Genetics and  
Pathogenesis of the Clostridia

PRINCIPAL INVESTIGATOR: Richard W. Titball, Ph.D.

CONTRACTING ORGANIZATION: Tufts University  
Boston, Massachusetts 02144

REPORT DATE: June 2003

TYPE OF REPORT: Final Proceedings

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;  
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20030520 051

# REPORT DOCUMENTATION PAGE

Form Approved  
OMB No. 074-0188

\*Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

<b>1. AGENCY USE ONLY</b> (Leave blank)		<b>2. REPORT DATE</b> June 2003	<b>3. REPORT TYPE AND DATES COVERED</b> Final Proceedings (26 Apr 03 - 30 Apr 03)	
<b>4. TITLE AND SUBTITLE</b> Fourth International Meeting on Molecular Genetics and Pathogenesis of the Clostridia			<b>5. FUNDING NUMBERS</b> DAMD17-02-P-0699	
<b>6. AUTHOR(S)</b> Richard W. Titball, Ph.D.				
<b>7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)</b> Tufts University Boston, Massachusetts 02144  E-Mail: rtitball@dstl.gov.uk			<b>8. PERFORMING ORGANIZATION REPORT NUMBER</b>	
<b>9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)</b> U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			<b>10. SPONSORING / MONITORING AGENCY REPORT NUMBER</b>	
<b>11. SUPPLEMENTARY NOTES</b> Original contains color plates: All DTIC reproductions will be in black and white.				
<b>12a. DISTRIBUTION / AVAILABILITY STATEMENT</b> Approved for Public Release; Distribution Unlimited				<b>12b. DISTRIBUTION CODE</b>
<b>13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information)</b> The 4 <sup>th</sup> International Meeting on the Molecular Genetics and pathogenesis of the Clostridia was held at the Woods Hole Marine Biological Laboratory during the period 26 <sup>th</sup> - 30 <sup>th</sup> April 2003. The aim of this meeting was to bring together scientists from around the world who are working on the pathogenic clostridia to discuss recent results and to facilitate discussions and the exchange of new information and ideas. 130 delegates from a range of countries attended the meeting. The meeting included sessions on Enterotoxins, Epidemiology Diagnosis and Treatment, Biotechnology and Medical Applications of Clostridial Biology, Membrane active toxins, Veterinary Diseases, Neurotoxins, Genomics, Mobile Elements and Genetics, Regulation of Virulence Gene Expression, Microbial Physiology and Pathogenesis and Host-Pathogen Interactions. The most recent research on toxins of significance to USAMRAA was presented at the meeting. An abstract booklet for this meeting has been provided to all delegates. The abstracts will also be published in the journal Anaerobe. Further details of the meeting are available at the web site <a href="http://w3.ouhsc.edu/cp2003/">http://w3.ouhsc.edu/cp2003/</a> . The financial support from USAMRAA was of critical value in allowing this meeting to take place, and this support has been acknowledged in all proceedings from the meeting and on the meeting web site.				
<b>14. SUBJECT TERMS</b> Clostridia, toxins, vaccines			<b>15. NUMBER OF PAGES</b> 71	
			<b>16. PRICE CODE</b>	
<b>17. SECURITY CLASSIFICATION OF REPORT</b> Unclassified	<b>18. SECURITY CLASSIFICATION OF THIS PAGE</b> Unclassified	<b>19. SECURITY CLASSIFICATION OF ABSTRACT</b> Unclassified	<b>20. LIMITATION OF ABSTRACT</b> Unlimited	

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)  
Prescribed by ANSI Std. Z39-18  
298-102

## Table of Contents

Cover.....	1
SF 298.....	2
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	9
Reportable Outcomes.....	9
Conclusions.....	9
References.....	9
Appendices.....	10

## Introduction

The 4<sup>th</sup> International Meeting on the Molecular Genetics and pathogenesis of the Clostridia was held at the Woods Hole Marine Biological Laboratory during the period 26<sup>th</sup> – 30<sup>th</sup> April 2003. The aim of this meeting was to bring together scientists from around the world who are working on the pathogenic clostridia to discuss recent results and to facilitate discussions and the exchange of new information and ideas. The topics of the meeting include some toxins which are considered to be potential biological warfare or bioterrorism agents (e.g. botulinum toxins and *C. perfringens* toxins). In this respect information on the mode of action, diagnostics and vaccines will be of significance to the mission of USAMRAA.

## Body

Program for the Fourth International Conference on the Molecular Biology and Pathogenesis of the Clostridia

*Saturday, April 26, 2003*

3:00-6:00 PM Registration (Swope 1)

5:00-6:00 PM Cocktail hour (Swope 2)

6:00-7:30 PM Dinner (Swope 2)

**8:00-9:15 PM Schering-Plough Animal Health Keynote Lecture (Lillie Hall) (Chair: Bruce McClane, USA)**

Julian Rood (Monash University, Clayton, Australia) *Clostridium perfringens*: From Plasmids to Pathogenesis

9:15-11:15 PM Schering-Plough Animal Health Reception (Meigs Room, Swope 2)

*Sunday, April 27, 2003*

7:00-8:30 AM Breakfast (Swope 2)

**8:30-10:30 AM Session I: Enterotoxins (Lillie Hall) (Chair: Monica Thelestam, Sweden)**

8:30-9:00 AM Klaus Aktories (Albert-Ludwigs University, Freiburg, Germany) Clostridial ADP-ribosylating toxins

9:00-9:20 AM Barbara Geric (University of Ljubljana, Ljubljana, Slovenia) The effect of *Clostridium difficile* binary toxin (CDT) in animals depends upon the animal model

9:20-9:40 AM Bradley G. Stiles (Toxinology Division, USAMRIID, Fort Detrick, MD USA) Some "complex" issues with *Clostridium perfringens* iota toxin

9:40-10:10 AM Bruce McClane (University of Pittsburgh School of Medicine, Pittsburgh, PA USA) The complex action of *Clostridium perfringens* enterotoxin

10:10-10:30 AM James G. Smedley (University of Pittsburgh, Pittsburgh, PA USA) A structure-function study of the *Clostridium perfringens* enterotoxin (CPE): Fine mapping of a region essential for cytotoxicity

10:30-11:00 AM Coffee break (Lillie lobby)

**11:00AM-1:00 PM Session II: Epidemiology, Diagnosis and Treatment (Chair: Tom Riley, Australia)**

11:00-11:25 AM Mark Wilcox (University of Leeds, Leeds, UK) Pathogenesis of *C. difficile* infection at the bedside

11:25-11:50 AM Stuart Johnson (VA Chicago Health Care System, Chicago, IL USA) Wide dissemination of toxin A-negative *Clostridium difficile* strains

11:50 AM-12:05 PM Thomas Åkerlund (Swedish Institute for Infectious Disease Control, Stockholm, Sweden) Relationship between *Clostridium difficile* strain type, toxin production in vivo and in vitro, and severity of disease in CDAD patients

12:05-12:30 PM Ciarán Kelly (Beth Israel Deaconess Medical Center, Boston, MA USA) Protective immunity and immunization

12:30-12:45 PM Sydney M. Finegold (VA Medical Center, West Los Angeles, CA USA) A novel, unusual *Clostridium* species common in human feces

12:45-1:00 PM Qiyi Wen (University of Pittsburgh School of Medicine, Pittsburgh PA USA) Development of a Duplex PCR Genotyping Assay for Distinguishing Between *Clostridium perfringens* Type A Isolates Carrying Chromosomal versus Plasmid-Borne Enterotoxin (*cpe*) Genes

1:00-2:30 PM Lunch (Swope 2)

2:30-3:30 PM Meeting of Organizing Committee and International Advisory Committee (Candle House 105)

4:30-6:30 PM Poster Session (Swope 2)

5:30-6:30 PM Cocktail hour (Swope 2)

6:30-7:45 PM Dinner (Swope 2)

**8:00-9:30 PM Session III: Biotechnology and Medical Applications of Clostridial Biology (Lillie Hall) (Chair: Amy Bryant, USA)**

8:00-8:30 PM Shibin Zhou (Johns Hopkins University, Baltimore, MD USA) Combination bacteriolytic therapy with clostridial spores plus anticancer chemotherapeutic agents.

8:30-8:50 PM Shie-Chau Liu (Stanford University, Palo Alto, CA USA) Tumor-specific enzyme/prodrug gene therapy using genetically engineered *C. sporogenes* as a gene delivery system combined with vascular-targeting agents

8:50-9:10 PM Michelle M. Merrigan (VA Chicago Health Care System, Chicago, IL USA) Prevention of *Clostridium-difficile*-Associated-Disease (CDAD) during continuous clindamycin administration: A clindamycin-resistant non-toxigenic *C. difficile* (CD) strain is effective, but is it safe?

9:10-9:30 PM Keiji Oguma (Okayama University Graduate School of Medicine and Dentistry, Okayama, Japan) Establishment of a new procedure for purifying fully activated *Clostridium botulinum* type B neurotoxin

*Monday April 28, 2003*

7:00-8:30 AM Breakfast (Swope 2)

**8:30-10:30 AM Session IV: Membrane-Active Toxins (Lillie Hall) (Chair: Michel Popoff, France)**

8:30-8:55 AM Alberto Alape-Giron (Universidad de Costa Rica, San Jose, Costa Rica) Role of surface glycoconjugates in the cellular sensitivity to *C. perfringens*  $\alpha$ -toxin

8:55-9:20 AM Yoshiro Ohno-Iwashita (Tokyo Metropolitan Institute of Gerontology, Tokyo, Japan) Perfringolysin O, a cholesterol-binding cytolysin, as a probe of lipid rafts

9:20-9:45 AM Rod Tweten (University of Oklahoma Health Science Center, Oklahoma City, OK USA) The structural biology of cholesterol-dependent cytolysins: the pathway to pore formation

9:45-10:10 AM Ambrose Cole (Birkbeck College/University of London, London, UK) Crystal structure of *C. perfringens*  $\alpha$ -toxin reveals the mode of action

10:10-10:30 AM Martha Hale (U.S. Army Medical Research Institute of Infectious Diseases, Frederick, MD) Detergent-resistant membrane microdomains facilitate Ib oligomerization and biological activity of *Clostridium perfringens* iota toxin

10:30-11:00 AM Coffee break (Lillie lobby)

**11:00AM-1:00 PM Session V: Veterinary Diseases (Lillie Hall) (Chair: Kristian Moller, Denmark)**

11:00-11:45 AM John Finnie (Institute of Medical and Veterinary Science, Adelaide, Australia) Pathogenesis of brain damage produced in sheep by *Clostridium perfringens* type D epsilon toxin

11:45 AM-12:15 PM Francisco Uzal (University of California-Davis, USA) Diagnosis of *Clostridium perfringens* infections in animals

12:15-12:30 PM Michael Waters (Oregon State University, Corvallis, OR USA) Genotyping and phenotyping of beta-2 toxigenic *Clostridium perfringens* fecal isolates associated with gastrointestinal disease in piglets

12:30-12:45 PM Stanley L. Marks (University of California, Davis, School of Veterinary Medicine, Davis, CA USA) Investigation of the role of beta-2 toxigenic *Clostridium perfringens* in canine diarrhea

12:45-1:00 PM Kevin Keel (University of Arizona, Tucson, AZ USA) Response of neonatal pigs to inoculation with *Clostridium difficile* spores or purified toxins A and B

1:00-2:30 PM Lunch (Swope 2)

**2:30-4:30 PM Veterinary Diseases Workshop (Candle House 104/105) (Chair: J. Glenn Songer, USA)**

Glenn Songer (University of Arizona, Tucson, AZ USA) Clostridia in Domestic Animals: Versatile, Powerful, Evolving

Kevin Keel (University of Arizona, Tucson, AZ 85721 USA) Ribotyping of *Clostridium difficile* from Pigs and Other Species

Francisco Uzal (California Animal Health and Food Safety Laboratory System, School of Veterinary Medicine, University of California, Davis, San Bernardino, CA USA) Clostridial diseases of animals in South America: Myth and Reality

Paul Hauer (Center for Veterinary Biologics, Ames, Iowa USA) An Overview of Clostridial Veterinary Biologics with an Eye Toward the Future

Question-and-answer period.

4:30-6:30 PM Poster Session (Swope 2)

5:30-6:30 PM Cocktail hour (Swope 2)

6:30-7:45 PM Dinner (Swope 2)

**8:00-10:10 PM Session VI: Genomics, Mobile Elements, and Genetics (Lillie Hall) (Chair: Tohru Shimizu, Japan)**

8:00-8:25 PM Ian Paulsen (The Institute for Genomic Research, Rockville, MD USA) Complete genome sequence of *Clostridium perfringens* strain ATCC 13124 and comparative genomics of the clostridia

8:25-8:50 PM Gerhard Gottschalk (Institute for Microbiology and Genetics, Göttingen, Germany) Insights in metabolism and toxin production from the complete genome sequence of *Clostridium tetani*

8:50-9:15 PM Nigel Minton (Center for Applied Microbiology and Research, Salisbury, UK) The development of *Clostridium difficile* genetic systems

9:15-9:40 PM Christoph von Eichel-Streiber (Johannes Gutenberg-University, Mainz, Germany) Variants of the ISTRon CdIST1 and the search for its ancestor

9:40-9:55 PM Marjon H. J. Bennik (Institute of Food Research, Norwich, UK) The genome sequence of proteolytic *Clostridium botulinum* ATCC 3502 (Hall A): Some highlights

9:55-10:10 PM Keiji Oguma (Okayama University Graduate School of Medicine and Dentistry, Okayama, Japan) Genome analysis of a *Clostridium botulinum* type C toxin-transducing phage

Tuesday April 29, 2003

7:00-8:30 AM Breakfast (Swope 2)

**8:30-10:30 AM Session VII: Neurotoxins (Lillie Hall) (Chair: Eric Johnson, USA)**

8:30-9:00 AM Giampietro Schiavo (Imperial Cancer Institute, London, UK) The journey of tetanus toxin in neurons

9:00-9:30 AM Michel Popoff (Institut Pasteur, Paris, France) Toxinogenesis in *Clostridium botulinum* and *Clostridium tetani*

9:30-9:45 AM Yukako Fujinaga (Okayama University, Okayama, Japan) Identification of functional subunits of *C. botulinum* type C 16S toxin involved in binding to intestinal epithelial cells and erythrocytes

9:45-10:00 AM Omar Qazi (Imperial College of Science, Technology and Medicine, London, UK) Two distinct binding sites on the tetanus toxin H<sub>C</sub> fragment are essential for ganglioside binding

10:00-10:15 AM Ornella Rossetto (Università di Padova, Padova, Italy) Control of the metalloproteolytic activity of clostridial neurotoxins

10:15-10:30 AM Bal Ram Singh (University of Massachusetts, Dartmouth, MA USA) Current and unsettled issues in molecular steps in botulinum toxin action

10:30-11:00 AM Coffee break (Lillie lobby)

**11:00AM-1:00 PM Session VIII: Regulation of Virulence Gene Expression (Lillie Hall) (Chair: Linc Sonenshein, USA)**

11:00-11:30 AM Tohru Shimizu (Tsukuba University, Tsukuba, Japan) Global regulatory networks and virulence genes in *Clostridium perfringens*

11:30-11:45 AM Jackie Cheung (Monash University, Clayton, Australia) Effect of VirR Box mutations on the expression of *pfoA*

11:45 AM-12:00 PM Sheena McGowan (Monash University, Clayton, Australia) Analysis of the DNA binding domain of the VirR response regulator from *Clostridium perfringens*

12:00-12:30 PM Bruno Dupuy (Institut Pasteur, Paris, France) Mechanism of toxin regulation in *Clostridium difficile*

12:30-12:45 PM Revathi Govind (TTUHSC, Lubbock, TX) Bacteriophage-mediated toxin regulation in *Clostridium difficile*

12:45-1:00 PM Glen Carter (University of Nottingham, Nottingham, UK) Analysis of the *Clostridium difficile* LuxS signalling system

1:00-2:30 PM Lunch (Swope 2)

6:30-7:30 PM Cocktail hour (Swope 2)

7:30-9:00 PM Banquet (Swope 2)

9:00-11:00 PM Party (Meigs Room, Swope 2)

*Wednesday April 30, 2003*

7:00-8:30 AM Breakfast (Swope 2)

**8:30-10:30 AM Session IX: Microbial Physiology and Pathogenesis (Lillie Hall) (Chair: Nigel Minton, UK)**

8:30-8:55 AM Elizabeth Furrie (University of Dundee, Dundee, UK) Swarming and toxin formation in *Clostridium septicum*

8:55-9:20 AM Peter Dürre (University of Ulm, Ulm, Germany) Regulation of sporulation and its relationship to secondary metabolism

9:20-9:45 AM Linc Sonenshein (Tufts University, Boston, MA USA) Stationary phase, sporulation and pathogenesis of *Clostridium difficile*

9:45-10:00 AM Mahfuzur R. Sarker (Oregon State University, Corvallis, OR USA) Characterization of *spo0A* homologue in enterotoxigenic *Clostridium perfringens* type A

10:00-10:15 AM Jeralyn D. Haraldsen (Tufts University, Boston, MA USA) Efficient sporulation of *Clostridium difficile* requires disruption of the  $\sigma^K$  gene

10:15-10:30 AM Hubert Bahl (University of Rostock, Rostock, Germany) Phosphate limitation in *Clostridium acetobutylicum*

10:30-11:00 AM Coffee break (Lillie lobby)

**11:00 AM-1:00 PM Session X: Host-Pathogen Interactions (Lillie Hall) (Chair: Rick Titball, UK)**

11:00-11:25 AM Amy Bryant (VA Medical Center, Boise, ID USA) Activation of gpIIBIIIa by *Clostridium perfringens* PLC ( $\alpha$ -toxin)

11:25-11:45 AM Shigeru Miyata (Kagawa Medical University, Kagawa, Japan) The molecular mechanism of *Clostridium perfringens* epsilon toxin cytotoxicity

11:45 AM - 12:00 PM Osamu Matsushita (Kagawa Medical University, Kagawa, Japan) Collagen-binding domain of clostridial collagenase genetic evolution and structure-function relationships

12:00-12:15 PM Ganes Chakrabarti (University of Pittsburgh School of Medicine, Pittsburgh, PA USA) Death pathways activated in Caco-2 cells by *Clostridium perfringens* enterotoxin

12:15-12:30 PM Stephen B. Melville (Virginia Polytechnic Institute and State University, Blacksburg, VA USA) Persistence of *Clostridium perfringens* in host tissues: Life inside a macrophage

12:30-12:45 PM Neil Fairweather (Imperial College, London, UK) S-layer and related surface proteins of *Clostridium difficile*: Molecular and functional properties and possible roles in virulence

12:45-1:00 PM Dennis L. Stevens (VA Med Center, Boise, ID USA) Immunization with C-domain of alpha toxin prevents lethal infection, localizes tissue injury and promotes host response to challenge with viable *Clostridium perfringens*



1:00-2:30 PM Lunch (Swope 2)

ABSTRACTS OF ORAL PRESENTATIONS – see appendix A

### **Key Research Accomplishments**

- The 4<sup>th</sup> International Meeting on the Molecular Genetics and pathogenesis of the Clostridia was held at the Woods Hole Marine Biological Laboratory during the period 26<sup>th</sup> – 30<sup>th</sup> April 2003.
- 130 delegates from a range of countries attended the meeting.
- The meeting included sessions on Enterotoxins, Epidemiology Diagnosis and Treatment, Biotechnology and Medical Applications of Clostridial Biology, Membrane active toxins, Veterinary Diseases, Neurotoxins, Genomics, Mobile Elements and Genetics, Regulation of Virulence Gene Expression, Microbial Physiology and Pathogenesis and Host-Pathogen Interactions.
- The most recent research on toxins of significance to USAMRAA was presented at the meeting.
- The financial support from USAMRAA was of critical value in allowing this meeting to take place, and this support has been acknowledged in all proceedings from the meeting and on the meeting web site.

### **Reportable Outcomes**

- An abstract booklet for this meeting has been provided to all delegates.
- The abstracts will also be published in the journal *Anaerobe* in autumn 2003.
- Further details of the meeting are available at the web site <http://w3.ouhsc.edu/cp2003/>.

### **Conclusions**

The 4<sup>th</sup> International Meeting on the Molecular Genetics and pathogenesis of the Clostridia was held at the Woods Hole Marine Biological Laboratory during the period 26<sup>th</sup> – 30<sup>th</sup> April 2003. 130 delegates from a range of countries attended the meeting. The meeting included sessions on Enterotoxins, Epidemiology Diagnosis and Treatment, Biotechnology and Medical Applications of Clostridial Biology, Membrane active toxins, Veterinary Diseases, Neurotoxins, Genomics, Mobile Elements and Genetics, Regulation of Virulence Gene Expression, Microbial Physiology and Pathogenesis and Host-Pathogen Interactions. The most recent research on toxins of significance to USAMRAA, and especially in the field of the mode of action, diagnostics and vaccines was presented at the meeting. A Veterinary Diseases Workshop was also held during the meeting. An abstract booklet for this meeting has been provided to all delegates. The abstracts will also be published in the journal *Anaerobe* during autumn 2003. Further details of the meeting are available at the web site <http://w3.ouhsc.edu/cp2003/>. The financial support from USAMRAA was of critical value in allowing this meeting to take place, and this support has been acknowledged in all proceedings from the meeting and on the meeting web site. The 5<sup>th</sup> International Meeting on the Molecular Genetics and pathogenesis of the Clostridia will be held in the UK in spring or summer of 2006.

### **References**

None

## **Appendix A**

### **ABSTRACTS OF ORAL PRESENTATIONS**

Saturday, April 26, 2003 8:00-9:15 PM Keynote Address

**CLOSTRIDIUM PERFRINGENS: FROM PLASMIDS TO PATHOGENESIS.**

J. I. Rood

Bacterial Pathogenesis Research Group, Department of Microbiology,  
Monash University, Victoria 3800, Australia.

*C. perfringens* is the causative agent of human gas gangrene, food poisoning, enteritis necroticans and non-foodborne diarrhoea. Our understanding of the genetics and pathogenesis of this pathogen has increased dramatically over the past two decades. We have gone from not knowing anything about the genetics of the organism to having the complete genome sequence and being able to use reverse genetics to study pathogenesis. Initial studies in this research group involved the comparative molecular analysis of R plasmids of *C. perfringens*. These results led to the identification of a novel mobilisable transposon, Tn4451, whose transposition is solely dependent upon a large resolvase type of site-specific recombinase and to the cloning of several antibiotic resistance determinants. These genes were subsequently used for the construction of shuttle vectors that are now almost universally used in *C. perfringens* genetics. The development of electroporation and transposon mutagenesis methodology has enabled major advances to be made in studying the role of extracellular toxins in the pathogenesis of gas gangrene and in identifying and analysing the regulatory network that controls toxin production in *C. perfringens*. By making single and double  $\alpha$ -toxin (*plc*) and perfringolysin O (*pfoA*) mutants and their complemented derivatives it has been shown that  $\alpha$ -toxin is essential for clostridial myonecrosis and that these toxins act synergistically in the disease process. The VirS/VirR two-component signal transduction system has been identified and shown to regulate toxin production. The response regulator VirR was shown to directly activate *pfoA* by binding to a pair of imperfect direct repeats, termed VirR boxes, that are located immediately upstream of the promoter. The N-terminal domain of VirR contains the conserved FxRxHrS motif, which has been shown to be essential for DNA binding. In summary, the development of *C. perfringens* genetics has resulted in a significant advancement of knowledge of how antibiotic resistance genes can move in *C. perfringens*, how this bacterium causes disease and how the expression of its toxic virulence factors is regulated.

Sunday, April 27, 2003 8:30-10:30 AM Session I: Enterotoxins

## CLOSTRIDIAL ADP-RIBOSYLATING TOXINS

K. Aktories, D. Blöcker, C. Wilde and H. Barth

Institute of Experimental and Clinical Pharmacology and Toxicology,  
University of Freiburg, D-79104 Freiburg, Germany.

Several species of the genus *Clostridium* produce ADP-ribosylating (ADP-r.) toxins that are divided into two groups: the Rho-ADP-r. exoenzymes (C3-like transferases) and the binary actin-ADP-r. toxins. The C3-like transferases are produced by *C. botulinum* types C and D, *C. limosum*, as well as *Bacillus cereus* and *Staphylococcus aureus*. These exoenzymes do not possess a specific binding and transport unit. They specifically modify RhoA, B and C at Asn41, which blocks the biological functions of Rho (e.g., regulation of the actin cytoskeleton, cell motility, cell cycle control and transcriptional activation).

Binary actin-ADP-r. toxins consist of an ADP-ribosyltransferase, and a cell binding and translocation component, the latter transfers the enzyme component into the cytosol. At least 5 toxins belong to this group: *C. botulinum* C2 toxin, *C. perfringens* iota toxin, *C. spiroforme* toxin, *C. difficile* transferase and the vegetative insecticidal protein VIP from *Bacillus cereus*. The binding and translocation components of these toxins are activated by proteolysis to release a N-terminal fragment, thereby allowing homoheptameric assembly. Depending on the toxin type, binding to target cells occurs before or after oligomerization. The enzyme component binds to the heptamers and this receptor-toxin complex is endocytosed. In an acidic endosomal compartment, the heptamer inserts into the membrane, allowing the translocation of the enzyme component into the cytosol. In the cytosol, the enzyme component ADP-ribosylates G-actin at arginine-177. This modification inhibits polymerization of monomeric actin, ultimately causing depolymerization of F-actin. Recently, detailed insights into the structure-function relationships of these toxins and their up-take mechanisms have been obtained and will be discussed further.

THE EFFECT OF *Clostridium difficile* BINARY TOXIN (CDT) IN ANIMALS DEPENDS UPON THE ANIMAL MODEL

B. Geric<sup>1,2</sup>, M. Rupnik<sup>1</sup>, S. P. Sambol<sup>2</sup>, R. J. Carman<sup>4</sup>, M. Grabnar<sup>1</sup>, D. N. Gerding<sup>2,3</sup>, S. Johnson<sup>5</sup>

<sup>1</sup>Dept. of Biology, University of Ljubljana, Vecna pot 111, 1000 Ljubljana, Slovenia. <sup>2</sup>Northwestern University Medical School, Chicago, IL, USA. <sup>3</sup>VA Chicago HCS Lakeside Div, 400 East Ontario Street, Chicago, IL 60611, USA. <sup>4</sup>TechLab Inc., 1861 Pratt Drive, Blacksburg, VA 24060-6364, USA. <sup>5</sup>Hines VA and Loyola University Medical School, Maywood, IL, USA.

*Clostridium difficile* is an important human pathogen responsible for *C. difficile*-associated diarrhea (CDAD) and potentially lethal pseudomembranous colitis (PMC) in antibiotic-treated patients. Two large clostridial toxins (TcdA and TcdB) are recognized as the main virulence factors of this pathogen. Recently, some *C. difficile* strains have been shown to produce an additional toxin, binary toxin (CDT), an actin-specific ADP-ribosyltransferase. Most of the CDT-producing strains also produce TcdA and/or TcdB and, therefore, the role of CDT in human disease is not yet defined. We have recently discovered *C. difficile* strains that produce CDT alone and have tested these strains in two different animal models. *C. difficile* strains B1 (A+/B+/CDT-) and M3 or 11186 (A-B-CDT-) were used as the positive and negative controls, respectively. Groups of 10 clindamycin-treated hamsters challenged with 100 CFU of A-/B-/CDT+ strains CS1/6009, R11402 and IS58 became colonized, but did not develop diarrhea. However, trypsinized and concentrated supernatants from A-/B-/CDT+ strains R11402, CS1/6009 and AA1/3126 caused marked enterotoxic fluid responses in the rabbit ileal loop assay with volume-to-length ratios of > 1. The ileal loop results suggest possible involvement of binary toxin in the development of CDAD disease, but antibiotic-treated hamsters did not develop signs of CDAD despite colonizing the stools of the hamsters. The role of CDT in CDAD may be as an adjunct to toxins TcdA and TcdB.

## SOME "COMPLEX" ISSUES WITH *Clostridium perfringens* IOTA TOXIN

B. G. Stiles<sup>1</sup>, M. L. Hale<sup>1</sup>, J. C. Marvaud<sup>2</sup>, and M. R. Popoff<sup>2</sup>

<sup>1</sup>Toxinology Division, USAMRIID, Fort Detrick, MD 21702-5011. <sup>2</sup>Unite des Toxines Microbiennes, Institut Pasteur, 75724 Paris Cedex 15, France.

Iota toxin belongs to a fascinating family of binary toxins that include *Bacillus anthracis* anthrax and *Clostridium botulinum* C2. Iota toxin consists of two unlinked proteins designated as Ia ( $M_r \sim 47$  kD), which is an ADP-ribosyltransferase that modifies actin, and Ib ( $M_r \sim 81$  kD) that binds to the cell surface and facilitates Ia entry into the cytosol. Western blots revealed that Ib incubated with Vero cells at 37°C rapidly generated a cell-associated, SDS-insoluble oligomer of Ib ( $M_r > 220$  kD) within 15 sec that was still present 110 min after cell washing. Oligomerization of Ib was temperature dependent (evident at 25° or 37°, but not 4°C) and facilitated by a pronase-sensitive, cell-surface protein. The Ib complex generated  $\text{Na}^+/\text{K}^+$  permeable channels on Vero cells within 5 min that were blocked by Ia. Two Ib-specific monoclonal antibodies (Mabs) that recognize unique, neutralizing epitopes within residues 632-655 either inhibited Ib binding to cells and/or oligomerization, unlike non-neutralizing Mabs that bind within Ib residues 28-66. The biologically inactive Ib protoxin (Ibp;  $M_r \sim 98$  kD), which binds to Vero cells, did not oligomerize or form ion-permeable channels on cells and neither trypsin nor chymotrypsin treatment of cell-bound Ibp generated a large complex. Further evidence that Ib oligomerization is necessary for biological activity of Ib was provided by a resistant cell line (MRC-5), which bound to Ib but was devoid of a large Ib complex. In summary, the biological activity of iota toxin is dependent upon Ib interactions with a protein receptor and rapid generation of a long-lived, cell-associated complex that forms ion-permeable channels in cell membranes.

## THE COMPLEX ACTION OF *CLOSTRIDIUM PERFRINGENS* ENTEROTOXIN.

B.A. McClane, U.Singh, G. Chakrabarti, and R. Trivedi

Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261, USA

*C. perfringens* enterotoxin (CPE) is a cytotoxin that forms a series of complexes in mammalian plasma membranes, including an SDS-sensitive small complex (~90 kDa) and two SDS-resistant large complexes of ~155 and ~200 kDa. Since formation of the small complex appears to be necessary but insufficient for CPE action, we have recently focused our studies on better understanding the contribution of the large complexes to CPE action. Kinetic studies demonstrated that ~155 kDa complex formation precedes formation of the ~200 kDa complex and coincides with the onset of CPE-induced membrane permeability changes, i.e., the ~155 kDa complex appears to be primarily responsible for CPE-induced cytotoxicity. In the polarized human enterocyte-like CaCo-2 cell line, the basolateral surface was found to be more CPE-sensitive than the apical surface. That effect is due, in large part, to a greater presence of CPE receptors on the basolateral vs. apical surface of those cells. Western blot and immunoprecipitation studies of CaCo-2 cells treated on their apical surface with CPE showed interactions between the enterotoxin and both occludin and certain claudins, which are major components of the epithelial tight junction. Occludin was found by Western blotting to be present in the ~200 kDa complex, while co-immunoprecipitations showed that CPE interacts with certain claudins when localized in the ~155 kDa complex. These CPE interactions can promote internalization of tight junction proteins, thereby disrupting tight junction integrity. Results obtained following CPE treatment of other models suggest these tight junction changes may increase paracellular permeability. That effect, along with direct CPE-induced enterocyte damage, could contribute to the diarrhea of CPE-induced gastrointestinal disease of humans and animals.

## A STRUCTURE-FUNCTION STUDY OF THE *CLOSTRIDIUM PERFRINGENS* ENTEROTOXIN (CPE): FINE MAPPING OF A REGION ESSENTIAL FOR CYTOTOXICITY

J. G. Smedley, III, B. A. McClane.

Department of Molecular Genetics and Biochemistry, The University of Pittsburgh, Pittsburgh, Pennsylvania 15261

The enteropathogenic effects of *Clostridium perfringens* food poisoning, as well as certain non-food borne diarrheas caused by this bacterium, are mediated by the *C. perfringens* enterotoxin (CPE). CPE is expressed by <5% of all type A isolates and is produced during sporulation in the intestinal tract. After initial binding of the toxin to its receptor(s), CPE becomes associated with host proteins in an SDS-resistant complex of ~155 kDa. The formation of this "large" complex occurs concomitantly with plasma membrane permeability changes and cellular morphologic damage. An additional ~200 kDa large complex, which has been shown to include the tight junction protein occludin, can also be experimentally isolated after longer CPE treatment. Formation of this larger complex is associated with tight junction damage and destruction of intestinal villi. Previous deletion and point mutational analyses of CPE have identified a region in the N-terminal portion of CPE important for cytotoxicity. In the present study, site-directed mutagenesis was employed to introduce alanine substitution mutations at residues in this biologically important region of the toxin. These variant CPE's were recombinantly expressed, affinity-purified, and characterized for their performance in well-established CPE activity assays. All tested variants showed similar protein confirmation, as measured by trypsin digestions, and were also able to bind CaCo-2 cells. Variants rCPE<sub>D45A</sub> and rCPE<sub>K46A</sub> demonstrated no loss of large complex formation or cytotoxicity, however variants rCPE<sub>G47A</sub> and rCPE<sub>D48A</sub> were non-cytotoxic. In large complex formation experiments, rCPE<sub>D48A</sub> was unable to form large complex. Interestingly, rCPE<sub>G47A</sub> did demonstrate formation of the ~155 kDa complex. This variant represents the first identification of a CPE variant that forms large complex without being cytotoxic. This finding may have structural implications suggesting a post-binding physical change to the CPE complex. Taken together, results from these and ongoing studies will more finely map amino acid residues in this region important for CPE cytotoxicity and will also provide information leading the exact mechanism of action of CPE.



11:00AM-1:00 PM Session II: Epidemiology, Diagnosis and Treatment

**PATHOGENESIS OF *CLOSTRIDIUM DIFFICILE* INFECTION AT THE BEDSIDE.**

M. H. Wilcox

Department of Microbiology, The General Infirmary and The University of Leeds, Leeds LS2 9JT.

*Clostridium difficile* infection is an iatrogenic disease and is therefore at least partially preventable. Most detected cases are hospitalised patients, but there may be a significant burden of unrecognised *C. difficile* infection in the community accounting for up to 28% of laboratory detected cases. *C. difficile* diarrhoea is more common in older patients (~80% of diagnosed cases affect in-patients aged  $\geq 65$  years), but increasing age *per se* is not a risk factor for infection severity. Significant risk factors for severe *C. difficile* diarrhoea include functional disability, cognitive impairment, and recent endoscopy. Some broad spectrum antibiotics, including ureidopenicillins (e.g. piperacillin-tazobactam) and ciprofloxacin, are significantly less likely to induce *C. difficile* infection in comparison with cephalosporins such as cefotaxime. Assessing such antibiotic-related risks and the underlying aetiologies has been hindered by the lack of a non-animal model. We have used a triple-stage chemostat model of the human gut to investigate the behaviour of *C. difficile* and components of the normal gut flora in response to exposure to antibiotics and/or their metabolites. We believe that the gut model is a promising method for studying *C. difficile* pathogenesis in conditions analogous to the *in vivo* situation.

Effective control of *C. difficile* in the hospital requires both antibiotic control and prevention of environmental seeding and spread of the bacterium. Restriction of cephalosporin and/or clindamycin prescribing, and avoidance of excessive duration surgical antibiotic prophylaxis can be used to reduce nosocomial *C. difficile* infection. Feedback of data on rates of antibiotic prescribing and *C. difficile* infection should be the starting point to controlling hospital endemics. Infection control measures such as improved hand cleansing and use of tympanic or disposable thermometers have been demonstrated to be effective at reducing the incidence of nosocomial *C. difficile* infection. Surveillance data indicate that epidemic *C. difficile* strains exist, and that these can be widely distributed in the hospital environment. We have shown that the UK epidemic strain (PCR ribotype I) has several potential virulence markers, including increased ability to germinate and to sporulate. There is accumulating evidence that hypochlorite use is more effective than other agents at reducing the environmental burden of *C. difficile*, and indeed that persistence of some strains may be exacerbated by exposure to non-chlorine-containing cleaning agents.

## WIDE DISSEMINATION OF A TOXIN A-NEGATIVE *CLOSTRIDIUM DIFFICILE* STRAIN

S. Johnson

Department of Medicine, Hines VA Hospital, Hines, IL 60141 and the Stritch School of Medicine, Loyola University, Maywood, IL 60153, USA.

Pathogenic strains of *Clostridium difficile* (CD) produce two large clostridial toxins, toxins A and B, of which toxin A has been considered to be the primary virulence determinant because of the potent in vitro activity of purified toxin A in animal models. Toxin A-negative, B-positive (A-B+) CD strains have now been recognized in many countries and multiple reports have documented cases of A-B+ CD-associated disease (CDAD), despite lack of toxin A expression by these strains. Different genetic variants have been reported that have the same A-B+ phenotype. However, the most common variation involves two major changes in the toxin A gene, a 1.8-kb deletion within the repetitive region and a nonsense mutation in the 5' end that codes for a premature stop codon. This variant, referred to as toxinotype VIII, has been recovered from patients with CDAD on four different continents. *HindIII*-restriction endonuclease analysis (REA) is a highly discriminatory typing system for CD which is able to distinguish different REA Groups (CF and CG) and subgroups (REA types CF1-11 and CG1-3) among toxinotype VIII strains. REA type CF4 has been recovered from cases of CDAD, including fatal pseudomembranous colitis, in Illinois, Minnesota, and California within the U.S and from the U.K. and Canada. REA group CF strains have been responsible for clonal hospital outbreaks in Canada (CF4) and the Netherlands (CF11). REA group CG strains have been recovered from asymptomatic infants in Belgium, as well as from patients in the U.S. Although the virulence mechanisms of these A-B+ variants is not known, supernatant and purified variant toxin B causes an atypical cytopathic effect (cell rounding and detachment) in cell culture and a delayed transepithelial resistance drop in polarized cell monolayer tight-junction assays.

RELATIONSHIP BETWEEN *CLOSTRIDIUM DIFFICILE* STRAIN TYPE,  
TOXIN PRODUCTION *IN VIVO* AND *IN VITRO* AND SEVERITY OF  
DISEASE IN CDAD PATIENTS

T. Åkerlund<sup>1</sup>, B. Svenungsson<sup>2,4</sup>, K. Jalakas-Pörmull<sup>3</sup>, Å. Lagergren<sup>2</sup>, J. Struwe<sup>2,3</sup>, I. Persson<sup>1</sup> and L. G. Burman<sup>1</sup>.

<sup>1</sup>Department of Molecular Epidemiology and Biotechnology, Swedish Institute for Infectious Disease Control, <sup>2</sup>Division of Infectious Diseases and <sup>3</sup>Division of Clinical Bacteriology, Department of Medicine, Karolinska Institute, Huddinge University Hospital, Huddinge, and <sup>4</sup>Department of Communicable Disease Control and Prevention, Karolinska Hospital, Stockholm, Sweden.

The factors determining the severity of diarrhea in CDAD patients are not fully understood, although underlying diseases and host immune response contribute to the disease outcome. Toxin production by *Clostridium difficile* *in vitro* is high in media containing a low concentration of glucose and/or low amounts of specific amino acids, but little is known about the toxin yield *in vivo* and if certain strains lead to more severe symptoms. Here we investigated three groups of CDAD patient having < 3 diarrhea per day (n=45 patients), 3-10 (n=97) or > 10 per day (n=22). PCR ribotyping of the patient strains showed that no specific PCR ribotype was associated with the disease outcome. The median toxin level from 60 consecutive patient fecal samples from each group (n=20 in each group) was 0.50 U/g, 6.8 U/g, and 150 U/g, respectively, showing a correlation between toxin level and severity of disease. The number of males/females in these groups was 11/9, 11/9 and 3/17, with a median age of 62 (1-93), 73.5 (1-91) and 56.5 (1-80), respectively. Finally, there was no correlation between maximum toxin yield *in vitro* (48 h cultures in PY) and *in vivo* indicating that factors such as bacterial count and fecal nutrients determining toxin expression are important for the total toxin yield and hence disease outcome.

## PROTECTIVE IMMUNITY AND IMMUNIZATION

Ciarán P Kelly, M.D.

Department of Gastroenterology, Beth Israel Deaconess Medical Center,  
Harvard Medical School, Boston

A number of studies have found that many patients with *C. difficile* associated diarrhea (CDAD) mount a systemic antibody response to toxin A and/or toxin B. Our recent clinical studies indicate that a serum IgG response to toxin A may be associated with protective immunity.

In one study we found that asymptomatic carriers of toxigenic *C. difficile* show an increase in their serum IgG anti-toxin A levels within 3 days of colonization.<sup>1</sup> Colonized subjects with high serum IgG anti-toxin A levels were 48 fold less likely to develop CDAD than those with lower anti-toxin A antibody levels. Thus asymptomatic carriers appear to mount an anamnestic immune response to toxin A that is associated with protection against diarrhea.

In another study we observed that patients with a single episode of *C. difficile* diarrhea had significantly higher levels of serum IgM against toxin A by day 3 of their illness and significantly higher serum levels of IgG against toxin A on day 12, compared to patients who later had recurrent diarrhea.<sup>2</sup> After adjusting for other risk factors, patients with *C. difficile* diarrhea and a low level of IgG against toxin A had a 48-fold greater risk of recurrence. These two prospective studies indicate that the strength and the time course of the host immune response to toxin A play an important role in determining the clinical outcome of *C. difficile* infection.

Although, as yet the immune response to toxin B has not been correlated as strongly with specific clinical outcomes, toxin B is clearly immunogenic in humans and antibody responses to toxin B may also play a role in immune protection against CDAD.

A *C. difficile* vaccine containing formalin-inactivated toxins A and B has been developed and was highly immunogenic in humans.<sup>3</sup> This vaccine is now being evaluated for prevention of CDAD in high risk patients and to produce hyper-immune human polyclonal IgG for passive immunotherapy.

1. Kyne L, Warny M, Qamar A, Kelly CP. *New England Journal of Medicine* 2000;342:390-7.
2. Kyne L, Warny M, Qamar A, Kelly CP. *Lancet* 2001;357:189-93.
3. Kotloff KL, Wasserman SS, Losonsky GA, et al. *Infect.Immun.* 2001;69:988-95.

## A NOVEL, UNUSUAL CLOSTRIDIUM SPECIES COMMON IN HUMAN FECES

Sydney M. Finegold<sup>1,2,3\*</sup>, Yuli Song<sup>4</sup>, Chengxu Liu<sup>4</sup>, Denise R. Molitoris<sup>4</sup> and Maureen McTeague<sup>5</sup>

<sup>1</sup>Infectious Diseases Section (111 F), VA Medical Center West Los Angeles

<sup>2</sup>Department of Medicine, UCLA School of Medicine <sup>3</sup> Department of Microbiology, Immunology, and Molecular Genetics, UCLA School of Medicine <sup>4</sup> Research Service, VA Medical Center West Los Angeles <sup>5</sup> Clinical Microbiology Laboratory, VA Medical Center West Los Angeles. 11301 Wilshire Blvd. Los Angeles, CA 90073

Twelve strains of an obligately anaerobic, gram-positive, rod-shaped, spore-forming organisms isolated from human fecal specimens were characterized using phenotypic and molecular taxonomic methods. There were two colony types produced, one yellow, raised and dry and the other white, flat, and dry with irregular edges. The colony types bred true on repeated subculture, but the two types had 100% sequence similarity. Microscopically, this organism is unique by virtue of its very large size—1-1.5  $\mu\text{m}$  in width  $\times$  30  $\mu\text{m}$  in length. Comparative 16S rRNA gene sequencing showed that the 12 strains were genetically highly related to each other (displaying >99.5% sequence similarity) and represent a previously unknown sub-line within the *Clostridium* Cluster XI. Strains of the unidentified bacterium used carbohydrates as fermentable substrates, producing acetic acid, isovaleric acid and PAA as the major products of glucose metabolism. The closest described species to the novel bacterium is *Clostridium glycolicum*, although a 16S rRNA sequence divergence of 4% demonstrated they are different species. The unknown bacterium was also readily distinguished from *Clostridium glycolicum* biochemically; it produces acid from mannitol and sucrose but not from xylose, whereas *C. glycolicum* fails to ferment mannitol or sucrose but produces acid from xylose. The novel bacterium does not produce alkaline phosphatase, in contrast to *C. glycolicum*. Based on phylogenetic and phenotypic evidence, it is proposed that the unknown fecal bacterium be classified as *Clostridium hypermegas*.

DEVELOPMENT OF A DUPLEX PCR GENOTYPING ASSAY FOR  
DISTINGUISHING BETWEEN *CLOSTRIDIUM PERFRINGENS* TYPE A  
ISOLATES CARRYING CHROMOSOMAL VERSUS PLASMID-BORNE  
ENTEROTOXIN (CPE) GENES

Qiyi Wen, Kazuaki Miyamoto, and Bruce A. McClane

Department of Molecular Genetics and Biochemistry, University of  
Pittsburgh School of Medicine, Pittsburgh PA 15261

About 5% of *Clostridium perfringens* type A isolates carry the gene (*cpe*) encoding *C. perfringens* enterotoxin. Those *cpe*-positive type A isolates are important causes of food poisoning and non-food-borne diarrheas in humans, as well as certain veterinary diarrheas. Previous studies have determined that the type A isolates causing both non-food-borne human gastrointestinal disease and veterinary disease carry their *cpe* gene on plasmids, while the type A isolates causing human food poisoning carry a chromosomal *cpe* gene. The current study reports the successful development of a duplex PCR assay that can rapidly genotype enterotoxigenic type A isolates (i.e., determine whether those *cpe*-positive isolates carry a chromosomal or plasmid *cpe* gene). For this purpose, recent sequencing results (Miyamoto et. al. 2002. Infect. Immun. 70:4261-4272) were used to design two primer sets that specifically amplify either a 3.3 kb PCR product from the plasmid *cpe* locus of type A isolates or a 2.1 kb PCR product from the chromosomal *cpe* locus of type A isolates. When these two primer sets were combined in a duplex PCR, they successfully discriminated between 27 chromosomal *cpe*, type A isolates vs. 5 plasmid *cpe*, type A isolates. The availability of this simple assay as a powerful investigative tool for diagnostic, epidemiologic and basic research purposes was then confirmed when it was successfully applied to investigate a recent *C. perfringens* food poisoning outbreak occurring in Oklahoma.

8:00-10:00 PM Session III: Biotechnology and Medical Applications of Clostridial Biology

COMBINATION BACTERIOLYTIC THERAPY WITH *CLOSTRIDIUM NOVI-NT* SPORES

S. Zhou<sup>1</sup>, L. Dang<sup>1</sup>, C. Bettegowda<sup>1</sup>, N. Agrawal<sup>1</sup>, I. Cheong<sup>1</sup>, L. Watson<sup>1</sup>, K. Kinzler<sup>1</sup>, B. Vogelstein<sup>1,2</sup>

<sup>1</sup>Sidney Kimmel Comprehensive Cancer Center and <sup>2</sup>Howard Hughes Medical Institute, Johns Hopkins Medical Institutions, Baltimore, Maryland 21231, USA.

The cores of most solid tumors are poorly vascularized which renders them hypoxic. The poor vascularization makes a significant fraction of the tumor inaccessible to chemotherapeutic drugs, whereas hypoxia diminishes the therapeutic efficacy of ionizing radiation that relies on oxygen. However, the poorly vascularized regions provide a perfect "breeding ground" for anaerobes. Thus, we screened a panel of anaerobes for their ability to proliferate in xenograft tumors. *Clostridium novyi* stood out in this screen which was subsequently rendered nonpathogenic by eliminating a residential phage that carries alpha-toxin and named *C. novyi-NT*. We tested the therapeutic efficacy of *C. novyi-NT* spores using xenograft tumor models in mice. The results indicated that *C. novyi-NT* is able to destroy a component of tumors that is not affected by traditional cancer therapy.

## TUMOR-SPECIFIC ENZYME/PRODRUG GENE THERAPY USING GENETICALLY ENGINEERED *C. SPOROGENES* AS A GENE DELIVERY SYSTEM COMBINED WITH VASCULAR-TARGETING AGENTS

S. C. Liu<sup>1</sup>, M. J. Dorie<sup>1</sup>, A. J. Giaccia<sup>1</sup>, A. Patterson<sup>2</sup>, W. Denny<sup>2</sup>, W. Wilson<sup>2</sup>, and J. M. Brown<sup>1</sup>.

<sup>1</sup>Department of Radiation Oncology, Stanford University, California, USA.

<sup>2</sup>Auckland Cancer Society Research Centre, University of Auckland, Auckland, New Zealand

Hypoxia is unique for human solid tumors and can be detrimental in cancer treatment. We have previously proposed that nonpathogenic spore-forming *Clostridia* can be used as a tumor targeting delivery system in gene therapy, because of their selective germination and proliferation in hypoxic/necrotic regions of solid tumors after intravenous injection of their inactive spore form, whereas germination does not occur in normal tissues (Lemmon *et al.* Gene Therapy 4:791-796,1997). We have demonstrated that spores of a genetically engineered *C. sporogenes* strain to express the *E. coli*-derived cytosine deaminase are effective in converting systemically injected nontoxic 5-fluorocytosine (5-FC) into the highly toxic anticancer drug, 5-fluorouracil (5-FU), thereby producing a tumor-specific antitumor activity (Liu *et al.* Gene Therapy 9: 291-296, 2002). However, 5-FU is active only against proliferating cells in tumors, thus is not a perfect anticancer agent. To explore other enzyme/prodrug systems, we have transformed *C. sporogenes* with another *E. coli*-derived enzyme, nitroreductase (NTR) a two-electron reductase that converts the dinitrobenzamide aziridine derivative CB 1954 into highly toxic bifunctional alkylating agents. Using the murine SCCVII and human HT29 carcinoma transplanted subcutaneously into C3H and nude mice, respectively, as our *in vivo* model systems. We have found no significant activity of the drug or the recombinant spores alone, but a major antitumor activity was observed with a single injection of NTR-producing *C. sporogenes* spores combined with CB 1954. We have further investigated the antitumor activity with the addition of the vascular-targeting agent DMXAA (5,6 dimethylxanthenone-4-acetic acid) to the combination of spores and dinitrobenzamide prodrugs. This is a highly potent combination that produces tumor cures with a single administration of these agents.



FUNCTIONAL CHARACTERIZATION AND THERAPEUTIC  
TARGETING OF CLAUDIN-4/CLOSTRIDIUM PERFRINGENS  
ENTEROTOXIN RECEPTOR IN PANCREATIC CANCER CELLS

P.Michl<sup>1</sup>, C.Barth<sup>1</sup>, M.Buchholz<sup>1</sup>, M.Rolke<sup>1</sup>, S.Kunsch<sup>1</sup>, B. McClane<sup>2</sup>, S.  
Tsukita<sup>3</sup>, G. Leder<sup>4</sup>, G. Adler<sup>1</sup> and T.M. Gress<sup>1</sup>

1) Department of Internal Medicine I, University of Ulm, Ulm, Germany, 2)  
Department of Molecular Genetics and Biochemistry, University of  
Pittsburgh School of Medicine, Pittsburgh, USA, 3) Department of Cell  
Biology, Faculty of Medicine, Kyoto University, Sakyo-ku, Kyoto, Japan, 4)  
Department of General Surgery, University of Ulm, Germany

**Background & Aims:** Recently, several membrane proteins of the claudin multi-gene family have been identified as integral constituents of tight junctions. In expression profiling approaches, we previously found claudin-4 to be overexpressed in pancreatic cancer. Since claudin-4 has been described to serve as a receptor for the cytotoxic *Clostridium perfringens* enterotoxin (CPE), we investigated the effect of CPE treatment on pancreatic cancer cells and the biological role of claudin-4 in pancreatic cancer cells. **Methods:** Expression of claudin-4 was analyzed by Northern blots. In vitro toxicity of CPE was determined by use of the trypan blue exclusion test and the

<sup>86</sup>Rubidium release assay. The effect of CPE on tumor growth and morphology was studied in Panc-1 xenografts. Claudin-4 was stably overexpressed in S2-007 pancreatic cancer cells showing only weak endogenous expression. Changes in proliferation, invasiveness and anchorage-independent growth were analyzed by thymidine incorporation assays, two-chamber invasion assays and soft agar assays. **Results:** Claudin-4 was overexpressed in the majority of tested pancreatic cancer tissues, pancreatic cancer cell lines and several other gastrointestinal tumors. In vitro, CPE led to an acute dose-dependent cytotoxic effect, restricted to claudin-4 expressing cancer cells, and dependent on the claudin-4 expression levels. In vivo, intratumoral injections of CPE in claudin-4 expressing nude mouse xenografts of the Panc-1 cell line led to large areas of tumor cell necrosis and significant reduction of tumor growth. Claudin-4 overexpression resulted in significantly reduced invasive and anchorage independent growth potentials of S2-007 cells. **Conclusions:** Claudin-4/CPER expression antagonizes invasiveness and anchorage-independent growth of pancreatic tumor cells. Targeting claudin-4/CPER expressing tumors with CPE represents a promising new treatment modality.

PREVENTION OF CLOSTRIDIUM-DIFFICILE-ASSOCIATED-DISEASE (CDAD) DURING CONTINUOUS CLINDAMYCIN ADMINISTRATION: A CLINDAMYCIN-RESISTANT NON-TOXIGENIC *C. DIFFICILE* (CD) STRAIN IS EFFECTIVE, BUT IS IT SAFE?

M. M. Merrigan<sup>1,2</sup>, S. P. Sambol<sup>1,2</sup>, S.B. Johnson<sup>3,4</sup>, D.N. Gerding<sup>1,2</sup>

<sup>1</sup> VA Chicago Health Care System, Lakeside Division, <sup>2</sup>Northwestern University, Chicago IL, <sup>3</sup>Hines VA Hospital and <sup>4</sup>Loyola University, Maywood, IL.

We propose to prevent *Clostridium-difficile*-associated-disease (CDAD) by intentionally colonizing hospital patients with non-toxigenic *C. difficile* (CD) during antibiotic treatment to prevent opportunistic colonization by toxigenic CD. In the animal model of CDAD, we have shown that hamsters given antibiotics followed by non-toxigenic CD are prevented from contracting lethal CDAD when challenged with toxigenic strains. Achieving non-toxigenic colonization during a multi-dose course of clindamycin is difficult, as most non-toxigenic CD are susceptible to clindamycin, whereas toxigenic CD strains are often resistant. We screened our CD collection and found a non-toxigenic strain, M13, which was resistant to clindamycin. One dose of strain M13 spores colonized hamsters during a continuous course of clindamycin, and prevented CDAD in 100% (15 of 15) of colonized animals challenged by 1 million spores of toxigenic strain B1, also clindamycin resistant. The mechanism of resistance to clindamycin in strain M13 and the potential for transfer of this resistance to other organisms are important issues if this strategy is to be employed for human disease prevention. Clindamycin resistance in epidemic toxigenic CD is mediated by a chromosomal *ermB* gene encoding a 23s ribosomal RNA methylase that conveys resistance to macrolides, lincosamides and streptogramins (MLS). Using PCR, we found that strain M13 and five related non-toxigenic strains also contain the *ermB* gene. Toxigenic *ermB* positive strains vary as to the presence of mobilizable element Tn5398, which has been demonstrated to transfer resistance to other bacterial species (Farrow et al Microbiology 2001;147:2717). It is not yet known if M13 contains any of the Tn5398 genes in addition to *ermB*. Further genetic analysis is in progress, but we will discuss the hypothetical use of strains such as M13 for CDAD prevention in the clinical setting, in the presence and in the absence of a mobilizable element such as Tn5398.

## ESTABLISHMENT OF A NEW PROCEDURE FOR PURIFYING FULLY ACTIVATED *CLOSTRIDIUM BOTULINUM* TYPE B NEUROTOXIN

K. Oguma, H. Arimitsu, K. Inoue, Y. Sakaguchi, J. Lee, Y. Fujinaga.

Department of Bacteriology, Okayama University Graduate School of Medicine and Dentistry, Okayama 700-8558, Japan

Type B strain produces 12S and 16S toxins, which are complexes of a neurotoxin (NTX) and a nontoxic component(s). The 12S toxin consists of a NTX and a nontoxic component that has no hemagglutinin (HA) activity, and the 16S toxin is formed by conjugation of 12S toxin with HA. We found that HA consists of four subcomponents designated HA-1, -2, -3a, and -3b, and that 16S toxin binds to lactose via HA1. Based on these findings and those 1) 16S toxin dissociates into a NTX and nontoxic components under an alkaline condition, and 2) the toxicity of NTX is fully activated by trypsin treatment at pH 6.0, we established an easy procedure for purifying the fully activated 16S and NTX by using a lactose gel column with different pH. The 12 S and 16 S toxins were precipitated with ammonium sulfate from the culture, and then partially purified by a cation-exchange column. Thereafter, 12 S and 16 S toxins were separated by using a beta-lactose gel column at pH 6.0; 12 S toxin passed through the column, whereas 16 S toxin bound to the column and eluted with lactose. The fully activated NTX was obtained by applying the trypsin-treated 16 S toxin on the same column at pH 8.0; NTX passed through the column, whereas remaining nontoxic components bound to the column. The toxicity of this purified fully activated NTX was retained for long period by addition of albumin in the preparation, indicating that this NTX preparation can be used for the treatment of dystonia.

Monday April 28, 2003 8:30-10:30 AM Session IV: Membrane-active toxins

**ROLE OF SURFACE GLYCOCONJUGATES IN THE CELLULAR SENSITIVITY TO *CLOSTRIDIUM PERFRINGENS* ALPHA-TOXIN.**

A. Alape-Girón<sup>1,2,3</sup>, M. Flores-Díaz<sup>1,2,3</sup>, G. Clark<sup>4</sup>, R. Titball<sup>4</sup>, and M. Thelestam<sup>3</sup>.

<sup>1</sup> Instituto Clodomiro Picado, Facultad de Microbiología, Universidad de Costa Rica, San José, Costa Rica; <sup>2</sup> Departamento de Bioquímica, Facultad de Medicina, Universidad de Costa Rica, San José, Costa Rica; <sup>3</sup> Microbiology and Tumor Biology Center, Karolinska Institutet, Stockholm, Sweden; <sup>4</sup> Defence Evaluation and Research Agency, CBD Porton Down, Salisbury, Wilts, UK, SP4.

*Clostridium perfringens* phospholipase C, also called alpha-toxin, is the major virulence factor in the pathogenesis of gas gangrene. A Chinese hamster mutant cell line deficient in uridine diphospho-glucose (UDP-Glc) and hypersensitive to the cytotoxic effect of alpha-toxin was previously isolated. However, the molecular basis relating the UDP-Glc deficiency to cell sensitization to alpha-toxin has not been previously established. Since UDP-Glc is required in the synthesis of proteoglycans, N-linked glycoproteins, and glycosphingolipids, the role of these surface glycoconjugates in the cellular sensitivity to alpha-toxin was studied. Inhibition of proteoglycan or glycoprotein synthesis did not affect the cellular sensitivity to alpha-toxin, whereas inhibition of glycosphingolipid synthesis sensitized cells to this toxin. Remarkably, a mouse melanoma cell line deficient in gangliosides due to a mutation in the UDP-Glc:ceramide glucosyltransferase gene was found to be specifically hypersensitive to alpha-toxin. Gangliosides protected hypersensitive cells from the cytotoxic activity of alpha-toxin and prevented the membrane disrupting effect of alpha-toxin on artificial membranes. The protective effect increased with the sialic acid content of the ganglioside oligosaccharide chain. This work demonstrates that a deficiency of gangliosides renders cells more susceptible to the membrane damage caused by *C. perfringens* alpha-toxin, providing new insights toward understanding the factors which determine the cellular sensitivity to this toxin.

**PERFRINGOLYSIN O, A CHOLESTEROL-BINDING CYTOLYSIN, AS A PROBE OF LIPID RAFTS.**

Y. Ohno-Iwashita<sup>1</sup>, Y. Shimada<sup>1</sup>, A.A. Waheed<sup>1,\*</sup>, M. Hayashi<sup>1</sup>, M. Inomata<sup>1</sup>, M. Nakamura<sup>1</sup>, M. Maruya<sup>2</sup> and S. Iwashita<sup>3</sup>.

<sup>1</sup>Tokyo Metropolitan Institute of Gerontology, Tokyo, Japan. <sup>2</sup>Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan. <sup>3</sup>Mitsubishi Kagaku Institute of Life Sciences, Tokyo, Japan.

The role of cholesterol in membrane lipid rafts is one critical issue in cellular signaling in relation to various diseases. We have focused on the potential of perfringolysin O ( $\alpha$ -toxin), a cholesterol-binding cytolysin produced by *Clostridium perfringens*, as a probe for membrane cholesterol. Previously we prepared a protease-nicked and biotinylated derivative (BC $\alpha$ ) of perfringolysin O that causes no membrane lesion. BC $\alpha$  binds selectively to cholesterol in cholesterol-rich microdomains of cell membranes, the domains that fulfill the criteria of lipid rafts. Thus BC $\alpha$  can be used to detect cholesterol-rich lipid rafts. This is in marked contrast to filipin, another cholesterol-binding reagent, which binds indiscriminately to cell cholesterol. Biochemical and cytochemical analyses using BC $\alpha$  are now under way to detect molecular components localized in cholesterol-rich lipid rafts. Recently we prepared and characterized several truncated fragments of perfringolysin O, and demonstrated that the C-terminal domain, domain 4 (D4), possesses the same binding characteristics as BC $\alpha$ . BIAcore analysis showed that D4 binds specifically to cholesterol with the same binding affinity as the full-size toxin. Cell-bound D4 was recovered predominantly in detergent-insoluble, low-density membrane fractions where raft markers, such as cholesterol, flotillin and Src family kinases, are enriched, indicating that D4 also binds selectively to lipid rafts. Furthermore, an enhanced green fluorescent protein (EGFP)-D4 fusion protein was revealed to be useful for real-time monitoring of cholesterol in lipid rafts in the plasma membrane. In addition, the expression of EGFP-D4 in the cytoplasm might allow the investigation of intracellular trafficking of lipid rafts. Simultaneous visualization of lipid rafts in plasma membranes and inside cells might help in gaining a total understanding of their dynamic behaviors.

\*Present address: NIDDK/NIH, MD, USA

## THE STRUCTURAL BIOLOGY OF CHOLESTEROL DEPENDENT CYTOLYSINS: THE PATHWAY TO PORE FORMATION.

R. K. Tweten.

Department of Microbiology and Immunology, The University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73190, USA.

Perfringysin O (PFO) is an ancillary pathogenesis factor of *C. perfringens* and a member of the large family of cholesterol-dependent cytolysins (CDCs). PFO is secreted by *C. perfringens* as a soluble monomer, but ultimately makes the transition to the membrane where it forms large homo-oligomeric pores with a diameter of approximately 25 nm. A hallmark trait of the CDCs is the absolute dependence of their cytolytic mechanism on the presence of membrane cholesterol. PFO undergoes many secondary and tertiary structural transitions upon its entry into the membrane that only occur in the presence of cholesterol. Our current studies are centered on the role of domain 4 in anchoring PFO to the membrane and the role(s) of cholesterol in the mechanism of the CDCs. Recent studies have shown that the loops at the tip of the domain 4 beta-sandwich insert into the surface of the bilayer and function to anchor PFO to the membrane. Therefore, domain 4 functions as a platform from which the subsequent structural transitions occur that ultimately lead to the insertion of the domain 3 transmembrane beta-hairpins and pore formation. The role of cholesterol in the mechanism of the CDCs has also been re-examined and found to play a more complex role than previously understood. Current studies show that the loss of cholesterol results in the inhibition of pre-pore to pore conversion.

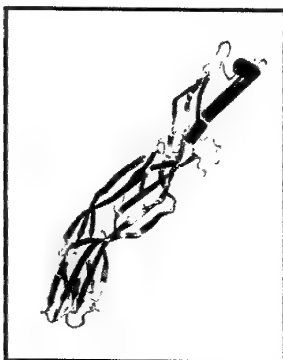
## CRYSTAL STRUCTURE OF EPSILON TOXIN REVEALS THE MODE OF ACTION

A.R. Cole<sup>1</sup>, M.R. Popoff<sup>3</sup>, D.S. Moss<sup>1</sup>, R.W. Titball<sup>2</sup>, A.K. Basak<sup>1</sup>

<sup>1</sup>School of Crystallography, Birkbeck College, London, WC1E 7HX.

<sup>2</sup>DSTL, Porton Down, Salisbury, SP4 0JQ.

<sup>3</sup>Centre National de Reference Anaerobies, Institut Pasteur, Paris



Of the many extracellular toxins of *C. perfringens*, epsilon toxin is thought to be the most potent. The strains responsible for producing epsilon toxin cause a very severe and often fatal form of enterotoxaemia. The toxin accumulates in the brain and to a lesser extent in the kidney causing widespread osmotic alterations by disrupting vascular endothelia. These degenerative changes eventually cause serum proteins and red blood cells to leak out of the vasculature and cause massive oedema. The action of epsilon toxin is thought to occur through the disruption

of cells by the formation of pores in cell membranes leading to the efflux of  $K^+$  ions. Various crystallographic methods have been undertaken to try and discover the three dimensional structure of this protein. Recently we have been able to ascertain the structure of epsilon toxin with the use of 'heavy atom' MAD techniques. This has made it clear that the overall fold of the protein bears a significant resemblance to that of aerolysin, a similar pore-forming toxin that shares many features with epsilon toxin. The overall structure of this toxin along with the similarities shared with aerolysin and the functional implications will be discussed in this presentation.

DETERGENT-RESISTANT MEMBRANE MICRODOMAINS  
FACILITATE Ib OLIGOMERIZATION AND BIOLOGICAL ACTIVITY  
OF *CLOSTRIDIUM PERFRINGENS* IOTA TOXIN

M. L. Hale<sup>1</sup>, J. C. Marvaud<sup>2</sup>, M. R. Popoff<sup>2</sup>, and B. G. Stiles<sup>1</sup>

<sup>1</sup>Toxinology Division, Department of Immunology and Molecular Biology,  
U. S. Army Medical Research Institute of Infectious Diseases, Frederick,  
Maryland 21702-5011; <sup>2</sup>Unite des Toxines Microbiennes, Institut Pasteur,  
Paris, France

Similar to *Bacillus anthracis* anthrax and *Clostridium botulinum* C2 toxins, *Clostridium perfringens* iota toxin contains two non-linked proteins, one that forms a large complex on toxin-susceptible cells that then facilitates cellular entry of the second, toxic subunit. *Clostridium perfringens* Iota b (Ib) complexes on the cell and forms a pore through which Iota a (Ia), a ribosyltransferase, enters the cytosol. At the present time, the cell receptor and membrane domains involved in Ib binding are unknown. In this study, Vero and MRC-5 cells were incubated with Ib (10 min, 37°C) and then extracted with cold Triton X-100. Immunoblot analysis of the separated cellular fractions showed that Ib oligomers localized in the detergent resistant membrane fraction (DRM). Iota toxin-resistant MRC-5 cells that bind Ib, but do not form oligomers, localized Ib in the soluble membrane fraction. Previous studies show that the Ib protoxin (Ibp) binds to Vero cells, but does not form cell-associated oligomers or facilitate iota cytotoxicity. Ibp was also primarily detected in the soluble membrane fraction and not in the DRM. Caveolin-1 and glycosylphosphatidylinositol-anchored glycoproteins were detected in the cholesterol-enriched DRM from Vero cells incubated with Ib and oligomerization was dramatically reduced when cells were pretreated with saponin, a glycoside that sequesters membrane cholesterol. Overall, these results suggested that cell-associated, biologically active Ib oligomers localize within DRMs.



11:00AM-1:00 PM Session V: Veterinary Diseases

**PATHOLOGY AND PATHOGENESIS OF BRAIN DAMAGE  
PRODUCED IN SHEEP BY *CLOSTRIDIUM PERFRINGENS* TYPE D  
EPSILON TOXIN.**

J. W. Finnie

Veterinary Services Division, Institute of Medical and Veterinary Science,  
Gilles Plains and Department of Pathology, University of Adelaide, South  
Australia, Australia

Microvascular endothelial damage by the epsilon toxin of *Clostridium perfringens* type D appears to be the fundamental cause of cerebral parenchymal injury and lesions occur in a seemingly dose- and time-dependent manner. Large doses of circulating toxin produce a severe, generalised, vasogenic cerebral oedema and an acute or peracute clinical course to death. With lower doses of toxin, or in partially immune sheep, focal necrosis, often bilaterally symmetrical, occurs in certain selectively vulnerable brain regions, which appear to become fewer as the toxin dose is reduced. These cases follow a more protracted clinical course, but death is the usual outcome. The precise pathogenesis of the focal brain damage found in subacutely intoxicated sheep is unresolved, but several possible mechanisms will be discussed.

## DIAGNOSIS OF *Clostridium perfringens* INTESTINAL INFECTIONS IN ANIMALS.

F.A. Uzal

California Animal Health and Food Safety Laboratory System, UC Davis,  
105 W. Central Ave., San Bernardino CA 92408, USA

*Clostridium perfringens* produces disease in several animal species, most of which are generically called enterotoxaemias. This microorganism can be a normal inhabitant of the intestine of most animal species including humans, but when the intestine is altered by sudden changes in diet, prolonged antibiotic therapy or other factors, *C. perfringens* proliferates in large numbers and produces several potent toxins that are absorbed into the general circulation or act locally with usually devastating effects on the host. History, clinical signs and gross post-mortem findings are useful tools in establishing a presumptive diagnosis of enterotoxaemias by *C. perfringens* in several animal species, although no definitive diagnosis of these diseases can be made without laboratory confirmation. Because all types of *C. perfringens* can be normal inhabitants of the intestine of most animals, culture of this microorganism from intestinal contents of animals has no diagnostic value, unless a colony count is performed and large numbers (usually more than  $10^6$  CFU/gr) of *C. perfringens* are found. The most accepted criterion to establish a definitive diagnosis of enterotoxaemia by *C. perfringens* is the detection of its toxins in intestinal contents. However, some of the major toxins of *C. perfringens* (i.e. epsilon toxin) can also be found, albeit in small amounts, in the small intestine of some animal species, which poses a diagnostic challenge. In these cases, the histopathology of the brain must be used as an alternative diagnostic tool, since the lesions produced by epsilon toxin in the brain of sheep, goats and probably cattle, are unique and pathognomonic for *C. perfringens* type D enterotoxaemia. Other auxiliary tests, such as measurement of urine glucose or observation of Gram stained smears of intestinal mucosa can also be used, and although they have presumptive diagnostic value when the results are positive they cannot be used to rule out a diagnosis of enterotoxaemia if the results are negative. In conclusion, the diagnosis of *C. perfringens* infections in animals is complex and it is more appropriate to rely on a combination of diagnostic techniques rather than on one single test.

GENOTYPING AND PHENOTYPING OF BETA2-TOXIGENIC  
*CLOSTRIDIUM PERFRINGENS* FECAL ISOLATES ASSOCIATED WITH  
GASTROINTESTINAL DISEASES IN PIGLETS.

M. Waters<sup>1</sup>, A. Savoie<sup>1</sup>, H. S. Garmory<sup>2</sup>, D. Buesche<sup>3</sup>, M. R. Popoff<sup>4</sup>, J. G. Songer<sup>3</sup>, R. W. Titball<sup>2</sup>, B. A. McClane<sup>5</sup> and M. R. Sarker<sup>1</sup>.

<sup>1</sup>Department of Microbiology, Oregon State University, Corvallis, OR 97331, USA. <sup>2</sup>Department of Biomedical Sciences, Dstl Chemical and Biological Sciences, Porton Down, Salisbury, SP4 0JQ, UK. <sup>3</sup>Department of Veterinary Sciences and Microbiology, University of Arizona, Tucson, AZ 95721, USA. <sup>4</sup>Unite des Toxines Microbiennes, Institute Pasteur, 75724 Paris, Cedex 15, France. <sup>5</sup>Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261, USA.

Although *Clostridium perfringens* is recognized as an important cause of clostridial enteric diseases, only limited knowledge exists concerning the association of particular *C. perfringens* toxinotypes (type A to E) with gastrointestinal (GI) diseases in domestic animals. Some *C. perfringens* isolates also produce the newly discovered beta2-toxin (CPB2). Recent epidemiological studies suggested that *C. perfringens* isolates carrying the gene (*cpb2*) encoding CPB2 are strongly associated with clostridial GI diseases in domestic animals, including necrotic enteritis in piglets and typhlocolitis in horses. These putative relationships, obtained by PCR genotyping, were tested in the current study by further genotyping and phenotyping 29 *cpb2*-positive *C. perfringens* pig GI disease isolates. Polymerase chain reaction and restriction fragment length polymorphism analysis reconfirmed the presence of *cpb2* gene sequences in all the disease isolates included in the study. Furthermore, pulsed-field gel electrophoresis genotyping analyses showed the pig GI disease isolates included in this study all carry a plasmid *cpb2* gene, yet no clonal relationships were detected between the surveyed *cpb2*-positive pig GI disease isolates. Finally, CPB2 Western blotting demonstrated CPB2 expression by all of the surveyed *cpb2*-positive isolates. The CPB2 made by five of these pig GI disease isolates was shown to have the same deduced amino acid sequence as the biologically-active CPB2 made by the original type C isolate CWC245. Collectively, our current results support a significant association between CPB2-positive *C. perfringens* isolates and diarrhea in piglets.

## INVESTIGATION OF THE ROLE OF $\beta$ 2-TOXIGENIC *CLOSTRIDIUM PERFRINGENS* IN CANINE DIARRHEA

S. L. Marks and E. J. Kather

Companion Animal Gastrointestinal Laboratory, Department of Medicine and Epidemiology, University of California, Davis, School of Veterinary Medicine, Davis, CA, 95616, USA.

*Clostridium perfringens* enterotoxin is thought to be the principle virulence factor involved in canine *C. perfringens*-associated diarrhea, and has been detected in 28-34% of diarrheic dogs. However, the potential role of other virulence factors needs further investigation. A recently described virulence factor, the  $\beta$ 2-toxin, has been associated with enterocolitis in piglets, and typhlocolitis in horses. A recently published study by Thiede et. al, 2001, evaluated the incidence of the  $\beta$  2-toxin gene (*cpb2*) in 24 dogs with severe diarrhea and reported 32% of the isolates were positive for the *cpb2* gene via PCR utilizing primer sequences outlined in Herholz et. al, 1999; however, no nondiarrheic dogs were evaluated. The principal objectives of this study were to determine the incidence of  $\beta$  2-toxigenic *C. perfringens* isolates in both diarrheic and non-diarrheic dogs, and to determine *in vitro* expression of the  $\beta$ 2-toxin gene. PCR was performed on 585 *C. perfringens* isolates obtained from 79 dogs (50 non-diarrheic and 29 diarrheic) for detection of the  $\beta$ 2-toxin gene using primer sequences described by Garmory et. al, 2000, that amplify a 567bp region of the gene. Initial PCR analysis on four *C. perfringens* isolates and our positive control utilizing the primer sequences published by Herholz et. al, failed to amplify the  $\beta$ 2-toxin gene. Sequencing results of the 567bp  $\beta$ 2 PCR product from one isolate indicated significant variation from published sequences. PCR results revealed 35 (70%) of the non-diarrheic dogs had at least one isolate positive for the  $\beta$ 2-toxin gene, whereas 19 (66%) of the diarrheic dogs had at least one isolate positive for the  $\beta$ 2-toxin gene. Western blotting was completed on 20  $\beta$ 2-positive isolates from diarrheic dogs (n=8) and non-diarrheic dogs (n=12) for detection of *in vitro* toxin production and revealed expression of the  $\beta$ 2-toxin gene in one isolate (13%) from a diarrheic dog and 2 isolates (17%) from non-diarrheic dogs. Twenty positive isolates will be subjected to a second PCR to amplify the entire coding region of the gene for sequence analysis due to the apparent variation in gene sequence between species. These preliminary findings suggest that the  $\beta$ 2-toxin does not appear to play a role in the pathogenesis of canine *C. perfringens*-associated diarrhea.

## CLOSTRIDIUM DIFFICILE SPORES OR PURIFIED TOXINS A AND B.

MK Keel<sup>1</sup>, KW Post<sup>2</sup>, and JG Songer<sup>1</sup>

<sup>1</sup>Department of Veterinary Science and Microbiology, University of Arizona, Tucson, AZ 85721 USA. <sup>2</sup>Rollins Animal Disease Diagnostic Laboratory, Raleigh, NC 27605 USA

*Clostridium difficile*-associated disease (CDAD) has become a common diagnosis in neonatal pigs in many swine-producing areas, in North America and elsewhere. As many as 60% of cases of neonatal enteritis include *C. difficile* as a possible etiologic agent and more than one-third of cases are of *C. difficile* infection in the absence of other likely agents of enteritis. Infected animals have typhlocolitis, with or without diarrhea, and mesocolonic edema; more severe cases may include hydrothorax and ascites. The disease has been reproduced by oral inoculation of colostrum-deprived piglets (Post KW, RD Glock, A Holtcamp, BH Jost, and J Glenn Songer. 2000. Reproduction of *Clostridium difficile*-associated enteritis by experimental inoculation of piglets. Amer Assoc Vet Lab Diagn. Birmingham, AL) but experimental disease has not yet been adequately characterized. We have further examined the etiopathology of porcine CDAD through inoculation of pigs with spores or toxins or exposure of porcine intestinal explants to toxin A. Intra-gastric inoculation of *Clostridium difficile*-free pigs with viable suspensions of spores caused signs and lesions consistent with the natural disease and with our earlier experimental findings. A mild typhlocolitis with multifocal necrosis of the superficial mucosa was evident microscopically. Day-old pigs gavaged with partially-purified toxins A and B (750  $\mu$ g or 250  $\mu$ g protein per pig) developed diarrhea within 3 h and became moribund in 10 h or less. Post mortem examination revealed effusion into the lumen of the small intestine and variable mesocolonic edema, hydrothorax, ascites and/or subcutaneous edema. Diffuse necrosis of the superficial mucosa was present in the small and large intestines. Toxin A purified by ion affinity chromatography (0.25 to 2.5  $\mu$ g/ml) was applied to intestinal explants, and a dose-dependent response was documented by light microscopic examination. Ultrastructurally, changes included swelling of endoplasmic reticulum and mitochondria, autophagy of organelles, loss of microvilli, and necrosis or apoptosis. In conclusion pigs are susceptible to oral inoculation of *C. difficile* and develop lesions similar to those observed in spontaneously occurring cases. Pigs are very sensitive to the toxins of *C. difficile* as indicated by the severe disease and rapid death effected by even small amounts of the combined toxins. This sensitivity was also reflected in the results of organ culture experiments involving inoculation with purified toxin A.

## RESPONSE OF NEONATAL PIGS TO INOCULATION WITH

2:30-4:30 PM Veterinary Diseases Workshop (Chair: J. Glenn Songer, USA)

**CLOSTRIDIA IN DOMESTIC ANIMALS: VERSATILE,  
POWERFUL, EVOLVING**

Glenn Songer  
Department of Veterinary Science and Microbiology, University of  
Arizona, Tucson, AZ USA

Dr. Songer will present an overview of clostridial disease in domestic animals, with emphasis on both persistent and emerging problems, and on opportunities.

## RIBOTYPING OF *CLOSTRIDIUM DIFFICILE* FROM PIGS AND OTHER SPECIES

Kevin Keel

Department of Veterinary Science and Microbiology, University of Arizona,  
Tucson, AZ USA

*Clostridium difficile*-associated disease (CDAD) has emerged as an important cause of economic loss for swine producers in North America and elsewhere in the world. Little is known of the epidemiology of CDAD, including the cycling of the organism among sows, piglets, and the swine-production environment. Differences in the size and copy number of the intergenic sequences separating the 16S and 23S ribosomal-RNA (rRNA) genes can be determined by examination of products of PCR amplification (PCR ribotyping). This has been used extensively as a typing method on which to base epidemiologic studies of human CDAD, and the method is acknowledged to be an effective and precise means of differentiating among a large number of strains. We applied PCR ribotyping to isolates obtained from piglets with CDAD. Template prepared from fresh cultures of *C. difficile* and primers specific for the 3' end of the 16S rRNA gene and the 5' end of the 23S rRNA gene were used in a standard PCR protocol. Products were examined by agarose gel electrophoresis. Results revealed that the majority of isolates (83%) were of a single PCR ribotype. The predominant ribotype among porcine isolates (arbitrarily designated Ribotype 1) is also the principal type found in calves with *C. difficile* infection. Thus, PCR ribotyping may find limited use as an epidemiologic tool for investigation of sources of *C. difficile* in cases of neonatal porcine typhlocolitis and the dissemination of isolates within farrowing units. Ribotype 1 is infrequently found in humans. Previous work examining the possible role of dogs and cats as sources of *C. difficile* for infection of humans revealed that strains causing disease in companion animals are genetically-distinct from those associated with human CDAD. Our findings in regard to piglet and calf strains concur with the results of these earlier studies. However, more detailed investigation, such as ribotyping of isolates from the gut of normal individuals engaged in swine production and from cases of CDAD in such persons, are warranted.

## **CLOSTRIDIAL DISEASES OF ANIMALS IN SOUTH AMERICA: MYTH AND REALITY**

Francisco Uzal

California Animal Health and Food Safety Laboratory System, School of Veterinary Medicine, University of California, Davis, San Bernardino, CA USA

Dr. Uzal will speak from his personal experience about clostridial diseases which are less-widely recognized in developed nations but remain significant problems in developing areas of the world. Differences arise due to nonuniform distribution of agents distinctive styles of management, and abundant opportunities for collaborative research and development span the range from basic to applied.

## **AN OVERVIEW OF CLOSTRIDIAL VETERINARY BIOLOGICALS WITH AN EYE TOWARD THE FUTURE**

Paul Hauer

Center for Veterinary Biologics, Ames, Iowa USA

Dr. Hauer will discuss the future of clostridial biologics for use in animals. He will address the potential for recombinant vaccines, and the associated problems and regulatory issues, and the pressure to decrease animal use in development and testing. Injection site reactions are a continuing problem, and he will present issues and potential for improvement. Dr. Hauer will also deal with specific needs for effective vaccines or diagnostic aids, with the need for improvements in existing vaccines, and the issue of duration of immunity.



8:00-10:00 PM Session VI: Genomics, Mobile Elements, and Genetics

**COMPLETE GENOME SEQUENCE OF *Clostridium perfringens* STRAIN ATCC 13124 AND COMPARATIVE GENOMICS OF THE CLOSTRIDIA**

I. T. Paulsen

The Institute for Genomic Research, Rockville, USA

*Clostridium perfringens* is the cause of the human diseases gas gangrene (myonecrosis), enteritis necroticans, acute food poisoning, and non-foodborne enterotoxemia. The complete genome sequence of *C. perfringens* ATCC13124, a human gas gangrene isolate has been determined, and sequencing efforts on the *C. perfringens* food poisoning strain SM101 are currently underway. Comparison of ATCC13124 with the published *C. perfringens* strain 13 has identified a variety of unique islands, including a large island of 250 kb partly derived from a phage insertion. Within the unique islands on these two strains, candidate genes have been identified that may play a role in the phenotypic differences observed in virulence, transformation and sporulation efficiency. Comparative genomics with other sequenced clostridial species and with other low GC Gram-positive organisms is facilitating the identification of a "core" set of clostridial genes.

## INSIGHTS IN METABOLISM AND TOXIN PRODUCTION FROM THE COMPLETE GENOME SEQUENCE OF *CLOSTRIDIUM TETANI*

G. Gottschalk<sup>1,2</sup> and H. Brüggemann<sup>1</sup>

<sup>1</sup>Göttingen Genomics Laboratory and <sup>2</sup>Department of General Microbiology, Institute of Microbiology and Genetics, Georg-August-University, D-37077 Göttingen, Germany

The complete genome sequence of *Clostridium tetani* E88, a variant of strain Massachusetts is presented. The genome consists of a 2,799,250-bp chromosome encoding 2,372 ORFs and a 74,082-bp plasmid containing 61 ORFs. The plasmid harbours the genes of the tetanus toxin and of a collagenase, which lacks domain II in comparison with collagenases from organisms such as *C. histolyticum* or *C. perfringens*. In addition, 7 regulatory proteins are encoded on plasmid pE88 including a two-component system. The genome contains a number of virulence-related genes including genes for tetanolysin, hemolysin III, Internalin A homologues and adhesion proteins. Of interest is a pathway reconstruction, which was done on the basis of genes present or absent. Most strikingly, *C. tetani* lacks a F<sub>o</sub>F<sub>1</sub>-ATPase; it contains several genes coding for enzyme systems, which generate a sodium ion gradient across the cytoplasmic membrane indicating that *C. tetani* is taking advantage of sodium ion gradients in order to carry out active transport processes. Results on the possible involvement of two-component systems in regulatory processes and on the function of an oxygenase will also be presented.

## THE DEVELOPMENT OF GENE SYSTEMS FOR CLOSTRIDIUM DIFFICILE.

N. P. Minton<sup>1,2</sup>, T. O'Keeffe<sup>2</sup>, M. Herbert<sup>2</sup>, M. Elmore<sup>2</sup>, G Carter <sup>1,2</sup>, I. Davis<sup>1</sup>, P. Williams<sup>1</sup> and D. Purdy<sup>2</sup>.

<sup>1</sup>Institute of Infections & Immunity, University of Nottingham, Queen's Medical Centre, Nottingham, NG7 2UH, UK; <sup>2</sup>Research Division, Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire, SP4 0JG, UK

Despite the increasing importance of *Clostridium difficile* in human disease, the organism remains poorly characterised, particularly with regard to virulence mechanisms. Significant insight is likely to accrue from the genome sequence, ([http://www.sanger.ac.uk/Projects/C\\_difficile/](http://www.sanger.ac.uk/Projects/C_difficile/)). However, to maximise the benefits of this data, effective gene transfer systems and vectors are urgently required.

Recently, we were able to show that plasmids based on an indigenous clostridial plasmid (pCD6) replicon could be introduced into 2 toxinogenic strains of *C. difficile* (CD3 and CD6) via *oriT*-based conjugative transfer from *Escherichia coli* donors (Purdy *et al.*, 2002, *Mol. Microbiol.* 46: 439-452). Transfer could not be demonstrated until the vectors employed were appropriately methylated, due to the presence of formidable restriction barriers. These studies have now been extended to the genome strain CD630. Through the evaluation of cloned recombinant products, we have shown that this strain possesses 5 distinct methylase genes, but no genes encoding cognate restriction enzymes. As a consequence, *oriT*-based plasmids are readily transferred to CD630 from standard *E. coli* donors using our previously developed conjugative procedure. These observations and evidence from elsewhere, suggests that the majority of clostridial methylases play no role in restriction/modification. Further refinements to our clostridial vectors are ongoing, including the development of integrational and expression vectors. The latter are being employed to investigate the production of antisense RNA as a means of modulating gene function.

**VARIANTS OF THE ISTRON CDIST1 AND THE SEARCH FOR ITS  
ANCESTOR**

**Christoph von Eichel-Streiber**

**Johannes Gutenberg-University, Mainz, Germany**

## GENOME ANALYSIS OF A *CLOSTRIDIUM BOTULINUM* TYPE C TOXIN-TRANSDUCING PHAGE

Y. Sakaguchi<sup>1)</sup>, T. Hayashi<sup>2)</sup>, K. Kurokawa<sup>3)</sup>, Y. Fujinaga<sup>1)</sup>, M. Ohnishi<sup>2)</sup>, K. Nakayama<sup>2)</sup>, H. Arimitu<sup>1)</sup>, K. Inoue<sup>1)</sup>, and K. Oguma<sup>1)</sup>

1) Department of Bacteriology, Okayama University Graduate School of Medicine and Dentistry, Okayama 700-8558. 2) Department of Microbiology, Miyazaki Medical College, Miyazaki 899-1692. 3) Genome Information Research Center, Osaka University Suita, Osaka 565-0871, Japan.

Genes for *Clostridium botulinum* type C toxin and C3 enzyme are carried by bacteriophages. A type C toxin-transducing phage, c-st, was re-isolated from a strain C-Stockholm, and determined the complete genome sequence. The genome was a linear double-stranded DNA with a G + C content of 26.2 %. The genome size was 185,681 bp. Identical 403-bp sequences were found at each end of the genome. We identified 210 potential protein coding regions (ORFs) on the genome, including genes for type C toxin and C3 enzyme, a number of genes related to DNA metabolism and genes homologous to those of a *Bacillus subtilis* bacteriophage, SP $\phi$ eta c $\phi$ . Many transposase-like genes were also identified. More intriguingly, most of the ORFs were bi-directionally oriented: genes on the left half of the genome were transcribed in the left to right direction, and those on the right half in the right to left. This feature, together with the result of GC nucleotide skew analysis, suggested that the phage replicates like a circular chromosome. Supporting this presumption, a circular form of c-st phage genome was detected in the phage lysogens by Southern blot analysis of their genomic DNA. On the genome structure of the passaged c-st phage (propagated successively many times on an indicator strain), extensive genome rearrangements were observed especially on its 5' and 3' terminal regions. The mechanism underlying the rearrangement will be discussed.

## THE GENOME SEQUENCE OF PROTEOLYTIC *CLOSTRIDIUM BOTULINUM* ATCC 3502 (HALL A): SOME HIGHLIGHTS

M.H.J. Bennik<sup>1</sup>, M. Mauchline<sup>2</sup>, F. Bosveld<sup>1</sup>, M. Elmore<sup>2</sup>, N.P. Minton<sup>2,3</sup>, J. Parkhill<sup>4</sup>, M.W. Peck<sup>1</sup>.

<sup>1</sup>Institute of Food Research, Norwich Research Park, Norwich NR4 7UA, UK, <sup>2</sup>Research Division, Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire, UK, <sup>3</sup>Institute of Infections & Immunity, University of Nottingham, Queen's Medical Centre, Nottingham, NG7 2UH, <sup>4</sup>Pathogen Sequencing Centre, The Wellcome Trust Genome Campus, Hinxton, Cambridge, UK

The anaerobic, Gram-positive, spore-forming bacterium *Clostridium botulinum* produces the highly toxic botulinum neurotoxins, which cause the paralytic disease, known as botulism. Three forms of botulism occur in humans: wound botulism, foodborne botulism and intestinal (infant) botulism. Foodborne botulism is caused by ingestion of foods containing pre-formed botulinum toxin (when spores germinate to render cells that produce toxin in a food before consumption), while the others are due to growth and toxin production by the organism in wounds or the GI tract. The capability to produce highly resistant spores may allow *C. botulinum* to survive harsh environmental conditions, including processing and preservation methods used in the food industry. Under favourable conditions, spores may germinate leading to vegetative cells that produce the neurotoxins and lead to disease. The genome of a representative strain of Group I, proteolytic *Clostridium botulinum* ATCC 3502 (Hall A), has been sequenced at the Sanger Institute in Cambridge ([www.sanger.ac.uk/Projects/C\\_botulinum](http://www.sanger.ac.uk/Projects/C_botulinum)). We will present preliminary findings from the genome sequence related to sporulation/germination and neurotoxin production. The genome sequence furthermore revealed the presence of a plasmid that is dedicated to the production of a bacteriocin, boticin.

Tuesday April 29, 2003 8:30-10:30 AM Session VII: Neurotoxins

## THE JOURNEY OF TETANUS TOXIN IN NEURONS

G. Schiavo<sup>1</sup>, S. Gschmeissner<sup>2</sup> and G. Lalli<sup>1</sup>

<sup>1</sup>Molecular NeuroPathobiology and <sup>2</sup>Electron Microscopy Laboratories, Cancer Research UK, London Research Institute, 44 Lincoln's Inn Fields, London WC2A 3PX, UK,

Retrograde transport is essential for neuronal homeostasis and survival. Pathogens and virulence factors, such as tetanus toxin (TeNT), exploit this pathway to reach their targets within the central nervous system. TeNT forms with botulinum neurotoxins (BoNTs), the clostridial neurotoxin family. At the neuromuscular junction, these neurotoxins bind with high affinity to lipid rafts, which contain their lipid and protein acceptors. After internalisation, BoNTs remain at the neuromuscular junction, whereas TeNT is transported to the neuronal cell body, where is transcytosed to inhibitory interneurons. The intracellular catalytic activity and targets of clostridial neurotoxins have been established but the molecular mechanisms controlling their neuronal binding and trafficking remain unclear.

To investigate the machinery controlling retrograde transport, we set up a time-lapse transport assay in living motor neurons using the carboxy-terminal fragment of TeNT (TeNT H<sub>C</sub>) as a probe. TeNT H<sub>C</sub> is rapidly endocytosed and transported in vesicular carriers of distinct size and speed. The average carrier speed overlaps with the retrograde transport speed observed for full length TeNT *in vitro* and *in vivo*. Retrograde transport of TeNT H<sub>C</sub> carriers lack typical endocytic markers and are not acidified during axonal transport. NGF and TeNT H<sub>C</sub> share the same retrograde transport carriers, which contain low-affinity neurotrophin receptor p75<sup>NTR</sup>. Retrograde transport of TeNT H<sub>C</sub> in motor neurons requires the integrity of microtubules and actin cytoskeleton. TeNT H<sub>C</sub> carriers rely on the non-redundant activities of dynein as well as kinesin family members. Moreover, actin-dependent myosin(s) are also necessary for retrograde movement. Immunofluorescence screening with isoform-specific myosin antibodies reveals significant colocalization of TeNT H<sub>C</sub> carriers only with myosin Va. Motor neurons from myosin Va <sup>-/-</sup> mice displayed slower TeNT H<sub>C</sub> retrograde transport compared to wild type cells. These findings suggest that coordination of myosin Va and microtubule-dependent motors is needed for fast axonal retrograde transport in motor neurons.

## REGULATION OF THE TOXINOGENESIS IN *CLOSTRIDIUM BOTULINUM* AND *CLOSTRIDIUM TETANI*.

M. R. Popoff, S. Raffestin, J. C. Marvaud, M. Gibert

Unité des Bactéries Anaérobies et Toxines, Institut Pasteur, Paris, France

*Clostridium botulinum* and *Clostridium tetani* produce potent neurotoxins. Botulinum neurotoxin (BoNT) is associated to non toxic proteins (ANTPs) to form high molecular weight complexes. In contrast, tetanus toxin (TeTx) does not form any complex. The genes encoding the ANTPs are clustered in the vicinity of *bont* genes in the *C. botulinum* toxinotypes. We have shown that in *C. tetani* the *tetx* gene is immediately preceded by a gene encoding a basic 21 kDa protein (TetR) which is conserved (*botR*) in *C. botulinum* strains. The *botR* gene is localized at the 5' extremity of the *botulinum* locus in *C. botulinum* C and D, and between the two divergent operons encoding the BoNT and ANTPs in *C. botulinum* A, B, F and G. Overexpression of *botR/A* in *C. botulinum* A, and *tetR* in *C. tetani*, induces an overproduction of BoNT/A, ANTPs, and TeTx respectively. Inversely, the partial inactivation of *botR/A* by a mRNA antisense method is accompanied by a decrease of BoNT/A and ANTPs production. *botR/A* regulates positively *bont/A* and also *antps* genes, whereas *tetR* seems to be only effective on *tetx* gene in *C. tetani*. *botR/A* and *botR/C*, transfected in *C. tetani*, increase the production of TeTx indicating that *C. botulinum* and *C. tetani* share a common regulation of the toxinogenesis. Specific mRNA assays show that BotR and TetR act at the transcriptional level. We have evidenced that, using crude bacterial lysate, BotR/A is able to form a complex with the promoter regions of the two divergent operons encoding the *bont* and *antps* genes. The same results were found with purified recombinant BotR/A in the presence of Core RNA polymerase from *E. coli*, whereas purified BotR/A only did not show any binding to DNA promoters. Sequence analysis indicates that BotR and TetR seem to recognize a common DNA site. BotR and TetR probably are part of the same family of alternative sigma factor required for the activation of toxin gene expression, which encompasses TxeR from *Clostridium difficile*.



IDENTIFICATION OF FUNCTIONAL SUBUNITS OF *C. BOTULINUM*  
TYPE C 16S TOXIN INVOLVED IN BINDING TO INTESTINAL  
EPITHELIAL CELLS AND ERYTHROCYTES

Y. Fujinaga<sup>1,2</sup>, H. Arimitsu<sup>1</sup>, Y. Sakaguchi<sup>1</sup>, J. Lee<sup>1</sup>, K. Inoue<sup>1</sup>, W. I. Lencer<sup>3,4</sup>, and K. Oguma<sup>1</sup>

<sup>1</sup> Dept. of Bacteriology, Okayama University Graduate School of Medicine and dentistry, 2-5-1 Shikata-cho, Okayama 700-8558, Japan. <sup>2</sup> PRESTO, Japan Science and Technology Corporation (JST), Japan. <sup>3</sup>GI Cell Biology, the Department of Pediatrics, Children's Hospital and Harvard Medical School, and the <sup>4</sup>Harvard Digestive Diseases Center, Boston, MA 02115

*C. botulinum* 16S and 19S progenitor toxin consist of a neurotoxin (NTX), a non-toxic non-HA (NTNH), and a hemagglutinin (HA). The HA consists of four distinct subcomponents designated HA1, HA2, HA3a, and HA3b. The nontoxic components (NTNH and HA) play a critical role in the food-borne botulism; the nontoxic components have a role of protecting the NTX against the attacks of acidity and proteases in the digestive tract. In addition, HA has been found to function as an adhesin of the 16S and 19S toxins to the microvilli of small intestine. To identify the adhesive subcomponent(s) of hemagglutinin (HA) of type C 16S toxin, all of the HA-subcomponents and some of their precursor forms were produced as recombinant proteins fused to glutathione S-transferase (GST). These proteins were evaluated for their capacity to adhere to human intestinal epithelial cells (T84 cells) and erythrocytes. GST-CHA1, GST-CHA3b, and GST-CHA3 (a precursor form of CHA3a and CHA3b) bound T84 cells and erythrocytes, whereas GST alone, GST-CHA2, and GST-CHA3a did not. Treatment of erythrocytes with neuraminidase completely abolished the adhesion activities of native type C 16S toxin, GST-CHA3b and GST-CHA3, but did not modify that of GST-CHA1. Based on these data and results of thin-layer chromatogram binding assay, it was concluded that the type C HA component contains two carbohydrate-binding subcomponents, CHA1 and CHA3b, which recognize carbohydrates in different specificities.

## TWO DISTINCT BINDING SITES ON THE TETANUS TOXIN H<sub>C</sub> FRAGMENT ARE ESSENTIAL FOR GANGLIOSIDE BINDING

O. Qazi and N. Fairweather

Centre for Molecular Microbiology and Infection, Imperial College of Science, Technology and Medicine, Flowers Building, Armstrong Road, London, SW7 2AZ

Tetanus toxin (TeNT) from *Clostridium tetani* consists of an N-terminal enzymatic L chain (50 kDa) disulphide bonded to a C-terminal (100 kDa) H chain, responsible for binding, trafficking and cytosolic escape of the L chain. H<sub>C</sub>, the 50kDa C-terminal fragment of the H chain, retains the binding and trafficking characteristics of whole toxin. Structural data suggests there are two distinct ganglioside-binding sites on H<sub>C</sub>; one binds Gal-GalNAc, the other binds sialic acid.

Mutant H<sub>C</sub> proteins containing amino acid substitutions within both sites were constructed and purified from *E. coli*. Proteins were assayed for binding to GT1b gangliosides and to N18 RE-105 neuroblastoma cells.

Our results confirm that both sites on H<sub>C</sub> are essential for binding ganglioside. GT1b binding of Gal-GalNAc site mutants is reduced to below 13% of wild type and binding of one mutant in the sialic acid is reduced to 10%, though it still binds cells. Surface plasmon resonance analysis indicates that H<sub>C</sub> does not bind ganglioside in a 1:1 stoichiometry and native PAGE suggests H<sub>C</sub> migrates as a tetramer. Pre-incubation of mutant proteins with GT1b and analysis by native PAGE demonstrated retardation of mutants with near wild type ganglioside binding, but not of mutant proteins.

## CONTROL OF THE METALLOPROTEOLYTIC ACTIVITY OF CLOSTRIDIAL NEUROTOXINS

O. Rossetto<sup>1</sup>, P. Caccin<sup>1</sup>, M. Rigoni<sup>1</sup>, E. Johnson<sup>2</sup>, G. Schiavo<sup>3</sup> and C. Montecucco<sup>1</sup>.

<sup>1</sup>Dipartimento di Scienze Biomediche Sperimentali, Università di Padova, V.le G. Colombo, 3, 35121, Padova, Italy. <sup>2</sup>Department of Food Microbiology and Toxicology, University of Wisconsin, Madison, Wisconsin, USA. <sup>3</sup>Laboratory of Neurobiopathology, Cancer Research Campaign, 44 Lincoln's Inn Fields, London WC2A, 3PX, UK

Botulinum neurotoxins (BoNTs, serotype A-G) and tetanus neurotoxins (TeNT), responsible for the clinical syndrome of botulism and tetanus respectively, are composed of two polypeptide chains joined by a single interchain disulphide bond. The heavy chain (H, 100 kDa) is responsible for the neurospecific binding and for the entry of the light chain (L, 50 kDa) into the neuronal cytosol. The L chains of the clostridial neurotoxins are zinc-dependent proteases very specific for three protein components of the neuroexocytosis apparatus (called SNARE proteins), whose cleavage results in a sustained blockade of the release of neurotransmitters at the synapse. TeNT, BoNT/B, /D, /F and /G cleave VAMP, a protein of the neurotransmitter-containing small synaptic vesicles; BoNT/A and /E hydrolyse SNAP-25, a protein of the cytosolic surface of the presynaptic membrane and BoNT/C cleaves both SNAP-25 and syntaxin, a protein present at presynaptic active zones, where neurotransmitter release takes place. Since this specific proteolytic activity is directed to different membrane proteins at specific peptide bond we investigated the influence of the lipid bilayer and its composition on this activity. *In vitro* we found that the rate of VAMP cleavage with all toxins tested is strongly enhanced, especially in the initial step of the reaction, by the presence of a lipid bilayer and this effect depends on lipid charge. Zinc-dependent protease activity of VAMP-specific neurotoxins likely involves the interaction between the L chain with the membrane of the target protein since the effect of lipids does not depend either on the presence of the H chain or on the presence of transmembrane domain of VAMP. The lack of effect of zwitterionic phospholipids and the salt inhibition indicate that electrostatic interactions play an important role in the lipid activation. The implication that this effect could have *in vivo* will be discussed.

## CURRENT AND UNSETTLED ISSUES IN MOLECULAR STEPS IN BOTULINUM ACTION

Bal Ram Singh

Department of Chemistry and Biochemistry, University of Massachusetts  
Dartmouth, Dartmouth, MA 02747, USA

The three major fields of botulinum toxin impact are food poisoning, bioweapons, and therapeutic applications. While there is a common biochemical mechanism involved in its action, routes of entry and possible molecular forms of the toxin could vary under the three impact conditions. The four-step molecular mechanism involved in botulism or botherapy (therapy with botulinum) are only partially understood. Relative importance of each of the steps in the biological effect of botulinum is critical for designing preventive and therapeutic measures against botulism or to prepare efficacious therapeutic product from botulinum agents. For example, neurotoxin associated proteins (NAPs) are known to protect the toxin from adversarial environmental conditions and increase its stability, and are also known to assist in the entry of the toxin across mucosal layer of the intestinal tract. NAPs also act as effective formulating agents for botulinum-based therapeutic reagents. How critical then NAPs are for botulism and botherapy? Similarly, how critical is the presence of a specific protein receptor at the nerve terminals? Available data on these and other steps involved in the biochemical manifestation of the botulinum toxin will be examined in this presentation. Supported by the U.S. Army Medical Research and Material Command under Contract No. DAMD17-02-C-001.

11:00AM-1:00 PM Session VIII: Regulation of Virulence Gene Expression

**GLOBAL REGULATORY NETWORKS AND VIRULENCE GENES IN *CLOSTRIDIUM PERFRINGENS*.**

T. Shimizu

Dept. of Infect. Biol., Inst. Basic Med. Sci., Univ. of Tsukuba, Japan

Recent whole genome sequencing of *C. perfringens* strain 13 revealed that the organism lacks many enzymes for amino acid biosynthesis and that it needs to obtain various essential materials from the host by producing several degradative enzymes and toxins, resulting in massive destruction of the host tissues. This indicates that the pathogenicity and nutritional acquisition must be highly coupled in *C. perfringens* infection. To examine the global regulatory network that governs the pathogenicity, transcriptome analysis was carried out using DNA microarrays for *C. perfringens*. The constructed DNA microarray contained approximately 3,000 spots including all 2,660 chromosomal genes and 63 plasmid-encoded genes. The total RNAs from the wild-type strain 13 and the *virR*-mutant strain TS133 were prepared at various growth phases, and were used as the labeling templates for cDNA synthesis using Cy5 and Cy3. After hybridization, the density of each spot was measured, and its difference between the wild-type and mutant strains was calculated. The expression of over 400 genes in *C. perfringens* genome were found to be affected by the VirR/VirS system, both positively and negatively. We also carried out the microarray experiment on the VR-RNA (secondary regulator for the VirR/VirS system) mutant, and found that approximately 350 genes were under the regulation of the VirR/VirS-VR-RNA cascade. Among these, many genes for transporters, PTS systems, degradative enzymes, toxins, and sugar-utilizing enzymes were found, indicating that the VirR/VirS system controls many genes for uptake and utilization of various nutrients as well as toxin genes.

## EFFECT OF VirR BOX MUTATIONS ON THE EXPRESSION OF *pfoA*.

J. K. Cheung and J. I. Rood

Bacterial Pathogenesis Research Group, Department of Microbiology,  
Monash University, Melbourne, Victoria 3800, Australia.

The VirS/R two-component signal transduction system is responsible for regulating many genes, including several toxin genes of *C. perfringens*. Previous work has shown that VirR directly activates perfringolysin O (PFO) production by binding to a pair of imperfect direct repeats, termed VirR boxes. These boxes are located immediately upstream of the promoter for the PFO structural gene, *pfoA*. Mutational analysis indicated that VirR binds independently at each of the boxes, such that the mutation of one binding site did not preclude binding to the other site. To examine what the effect these mutations would have on the expression of *pfoA* *in vivo*, constructs containing the various mutated VirR boxes upstream of *PpfoA* were introduced into a *C. perfringens pfoA* mutant, JIR 4228, and haemolysin assays were carried out. The results showed that mutation of either VirR box had the same effect on PFO production as the mutation of both VirR boxes. It is concluded that both VirR boxes are required for full transcriptional activation of *pfoA* and for wild-type PFO production. Thus, although VirR binds independently to each VirR box, there must be interaction between the bound proteins to activate gene expression. Potential protein-protein interactions were also observed when the spacing between the two boxes was altered. The centre of each VirR box is separated by 21 bp. This distance, which equates to two turns of the DNA helix, implies that the boxes are on the same face of the helix. To examine whether helical phasing is important in transcriptional activation, the VirR box regions were altered such that either a 5 bp or 10 bp insertion or a 5 bp deletion was made in the region separating the boxes. The resultant constructs were examined as before. Haemolysin assays showed that the PFO levels decreased to that of the vector control with the insertion or deletion of half a helical turn (5 bp) while the insertion of one helical turn (10 bp) severely reduced PFO production compared to wild-type. Interestingly, gel mobility assays demonstrated that purified His-tagged VirR could still bind to the altered VirR box regions. These results support the hypothesis that protein-protein interactions are required to activate gene expression.

# ANALYSIS OF THE DNA BINDING DOMAIN OF THE VirR RESPONSE REGULATOR FROM *CLOSTRIDIUM PERFRINGENS*

S. McGowan<sup>1</sup>, I. S. Lucet<sup>1</sup>, J. R. O'Connor<sup>1</sup>, J. K. Cheung<sup>1</sup>, M. M. Awad<sup>1</sup>, J. C. Whisstock<sup>2</sup> & J. I. Rood<sup>1</sup>

<sup>1</sup>Bacterial Pathogenesis Research Group, Department of Microbiology, and

<sup>2</sup>Department of Biochemistry and Molecular Biology, Monash University, Vic 3800, Australia.

The VirS/VirR two-component signal transduction system regulates the production of several extracellular toxins in *C. perfringens*, including *pfoA*, the structural gene for perfringolysin O. Purified His-tagged VirR activates *pfoA* by binding directly to two VirR boxes found immediately upstream of the *pfoA* promoter. Comparative sequence analysis of the 236 amino acid VirR protein revealed a two domain structure. The N-terminal domain had significant sequence similarity to response regulators of known structure, such as PhoB. The C-terminal domain did not have close sequence similarity to any protein of known structure. However, database searching identified two highly conserved regions: the FxRxHrS and the SKHR motifs. Random mutagenesis identified one further residue, a functionally important methionine (M172), located outside the conserved motifs. Site-directed mutagenesis of the two motifs showed that amino acids R186, H188, S190, S216, K217, H218 and R219 were essential for activity since even conservative substitutions in these positions resulted in essentially non-functional proteins. Seven mutant proteins including the random mutant M172V, were purified and shown to have a lower binding affinity for the VirR boxes, compared to the wild-type protein. It is concluded that the two VirR motifs are essential for proper DNA-protein interactions and that the entire C-terminal domain constitutes a novel DNA binding domain.

## MECHANISM OF TOXIN REGULATION IN *CLOSTRIDIUM DIFFICILE*

B.Dupuy<sup>1</sup>, N. Mani<sup>2</sup>, S. Matamouros<sup>1</sup>, S.T. Cole<sup>1</sup> and A.L. Sonenshein<sup>2</sup>.

1. Unité de Génétique Moléculaire Bactérienne, Institut Pasteur, Paris, France. 2. Department of Molecular Biology and Microbiology, Tufts University School of Medicine, Boston, MA 02111-1800.

Infection with *Clostridium difficile* can produce a wide spectrum of clinical manifestations ranging from mild diarrhea to potentially lethal pseudomembranous colitis (PMC). This variation in response seems to be due as much to host factors as to differential expression of virulence factors and especially genes for production of toxins A and B. However, the molecular mechanism(s) that triggers this transition is unknown. Toxin synthesis is growth phase-dependent and subject to multiple forms of environmental regulation. Thus, the transcription of the *tox* genes is turned on as a response to a shortage of carbon sources and certain amino acids. Moreover, it has been shown that temperature also controls toxin production at the level of transcription, with an optimum at 37°C. Expression of the *tox* genes is strongly dependent on TxeR, the product of the gene located upstream of the *toxB-toxA* cluster. By *in vivo* and *in vitro* transcription assays we have demonstrated that TxeR is an alternative RNA polymerase sigma factor that directs transcription from the *tox* promoters and its own promoter in response to physiological conditions tested. The relatedness of TxeR to BotR of *C. botulinum*, TetR of *C. tetani* and UviA of *C. perfringens* suggested that these regulators might be also alternative RNA sigma factors. We recently demonstrated that UviA is required, as a sigma factor, for the activation of the UV-inducible bacteriocin gene of *C. perfringens*. Moreover, we have shown by *in vivo* and *in vitro* transcription assays that UviA and TxeR are interchangeable. Because the sequences of these proteins bear little discernible resemblance to other members of the  $\sigma^{70}$  family, we suggest that TxeR and UviA are the first members of a novel sub-group (herein designated group 5) of the  $\sigma^{70}$  family.



## BACTERIOPHAGE MEDIATED TOXIN REGULATION IN CLOSTRIDIUM DIFFICILE.

R. Govind, J. A. Fralick and R. D. Rolfe.

Department of Microbiology and Immunology, TTUHSC, Lubbock, TX, 79430.

*Clostridium difficile* is the most important single identifiable cause of nosocomial antibiotic associated diarrhea and colitis. Virulent strains of *C. difficile* produce two large protein toxins, toxin A and toxin B that are involved in pathogenesis. During phage therapy experiments in which we employed bacteriophage, CD119, to target a virulent *C. difficile* strain, CD602, we isolated CD602 cells, which were resistant to this phage. Subsequently, we were able to determine that these "phage resistant" cells were actually CD119 lysogens. Interestingly, one of these lysogens produced approximately 3 times the amount of toxin A as did the parent strain CD602, as determined by ELISA. Furthermore, southern blot analysis of this lysogen indicated that the prophage had integrated into the bacterial chromosome and RT-PCR and northern analysis indicated that the expression of *toxA* and *toxB* were up regulated. Electron microscopic analysis of phage CD119 showed it to be a lambda-like in structure and biochemical analysis indicated that it contained a double-stranded DNA genome of approximately 40Kb. The genes for toxins A and B reside in a 19 kb pathogenicity locus (PaLoc) along with *txeR*, *tcdC* and *tcdE*. Genetic analysis of *TxeR* and *TcdC* suggest that they are involved in the positive and negative regulation of *tox A* and *tox B* expression, respectively. Computational analysis of the amino acid sequence of *tcdE* revealed structural features that are strikingly similar to a class of bacteriophage proteins called holins. However, thus far there is little evidence for phage-mediated regulation of toxin genes in *C. difficile*. We are currently sequencing the CD119 phage genome in an attempt to elucidate the mechanism by which CD119 lysogens of CD602 up-regulate *toxA* and *toxB*.

## ANALYSIS OF THE CLOSTRIDIUM DIFFICILE LUXS SIGNALLING SYSTEM

G. Carter<sup>1,2</sup>, D. Purdy<sup>2</sup>, Y. Mahida<sup>1</sup>, P. Williams<sup>1</sup>, N. Minton<sup>1,2</sup>

<sup>1</sup>Institute of Infections and Immunity, University of Nottingham, Queen's Medical Centre, Nottingham, NG7 2UH, UK <sup>2</sup>Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wilts, SP4 0JG, UK

*Clostridium difficile* is a multiply antibiotic resistant human pathogen, which is becoming increasingly difficult to treat. As a consequence, novel therapeutic agents are urgently needed. The attenuation of virulence through the blockade of bacterial cell-to-cell communication (quorum sensing) is one potential therapeutic target. To begin our exploration of quorum sensing and the pathogenicity of *C. difficile*, we focused on *luxS*/autoinducer-2 (AI-2) signalling.

Cell-free culture supernatants of *C. difficile* induced bioluminescence in a *Vibrio harveyi* AI-2 biosensor indicating that this Gram-positive pathogen produces AI-2 or a related signal molecule. The cloned *C. difficile luxS* gene complemented the AI-2 negative *E. coli* DH5 $\alpha$  *in trans* and restored AI-2 production. Analysis of the *C. difficile* genome database suggested that the *C. difficile luxS* homologue is part of an operon that includes two other genes, *metH* and *orfX*. Adjacent to *luxS* we have located a potential transcriptional regulator and sensor kinase. RT-PCR was used to confirm the genetic organization of the *luxS* locus, indicating transcriptional linkage between the response regulator and sensor kinase genes and between *luxS*, *metH* and *orfX*.

Since the construction of mutants by allelic exchange has so far not proved possible in *C. difficile*, we have developed an antisense strategy to inhibit expression of the *luxS* operon genes and putative response regulator. While we have so far been unable to block AI-2 production using antisense technology, AI-2 levels could be modulated by controlling expression of the putative transcriptional regulator. To our knowledge, this is the first reported use of antisense technology for the modulation of gene expression in *C. difficile*.

Wednesday April 30, 2003 8:30-10:30 AM Session IX: Microbial Physiology and Pathogenesis

**PHYSIOLOGIC STUDIES ON SWARMING AND TOXIN FORMATION IN *CLOSTRIDIUM SEPTICUM*.**

E. Furrie, S. Macfarlane and G. T. Macfarlane.

University of Dundee, MRC Microbiology and Gut Biology Group, Ninewells Hospital Medical School, Dundee DD1 9SY, UK.

*Clostridium septicum* is the aetiological agent of several diseases that affect humans and animals. Like a number of other clostridia, *Cl. septicum* can undergo cellular differentiation to form giant cells, that are capable of a form of multicellular behaviour known as swarming. Environmental and physiologic factors affecting swarm cell development in this organism were investigated using chemostats operated at varying dilution rates ( $D = 0.02 \text{ h}^{-1}$  -  $D = 0.65 \text{ h}^{-1}$ ), under different conditions of nutrient limitation. Cellular differentiation only occurred at low specific growth rates, indicating that the swarming phenomenon was, in part, a response to nutrient depletion. Differential expression of virulence factors was detected in swarm cells. For example, haemolysin was secreted by short rods, but not by swarm cells, whereas in C- and N-limited chemostats, only swarm cells synthesised DNase, hyaluronidase and neuraminidase. Both swarm cells, and short rods, were cytotoxic towards Vero cells. Mucin was chemotactic to *Cl. septicum*, and synthesis of mucin-degrading enzymes was catabolite regulated. Thus, glycosulfatase secretion only occurred in swarm cells, at low dilution rates in mucin-limited cultures. Measurements of mucin oligosaccharide uptake by *Cl. septicum* showed that N-acetyl glucosamine, N-acetyl galactosamine and galactose were the principal sources of C and energy in this glycoprotein. Neuraminic acid was not utilized, suggesting that neuraminidase synthesis does not have a direct nutritional role in this bacterium.

## REGULATION OF SPORULATION AND ITS RELATIONSHIP TO SECONDARY METABOLISM

P. Dürre, B. Schiel, M. Mönch, and C. Hollergschwandner

Mikrobiologie und Biotechnologie, Universität Ulm, 89069 Ulm, Germany

Bacteria of the genus *Bacillus* initiate endospore formation by activation of the so-called phosphorelay, in which phosphate is transferred from several sensor kinases via a chain of phosphotransfer proteins (Spo0F and Spo0B) to the essential sporulation response regulator Spo0A. All clostridia sequenced so far also possess a *spo0A* gene, but lack genes encoding *spo0F* and *spo0B*. Thus, a typical phosphorelay does not exist, and different signals seem to induce sporulation. In *Clostridium acetobutylicum*, *sigH* and *spo0A* genes are constitutively transcribed during growth. No increase of these transcripts occurs, when cells enter the stationary growth phase and initiate endospore formation. However, reporter gene studies revealed that activity controlled by the *spo0A* promoter can only be detected at the end of the exponential growth phase. Primer extension experiments indicated 3 transcription start points, two of which could also be the result of mRNA processing. One signal obviously stems from transcription off a *sigA*-dependent promoter, consistent with the constitutive expression observed. A Spo0A-binding motif (0A-box) was detected just upstream of the third signal, with adjacent palindromic sequences at either side.

Overexpression and purification of Spo0A in a pET-vector yielded very low amounts of the protein. Enhanced expression in a *B. subtilis* *spo0A* mutant is attempted. *In vitro* phosphorylation of Spo0A with acetyl phosphate was possible. Gel retardation experiments revealed binding to 0A-boxes upstream of the *spo0A*, *abr*, *spoIIG*, *adc*, and *sol* operons. The latter two encode genes responsible for acetone and butanol production. Targeted mutation of the control region sequences indicated the involvement of additional transcription factors, acting in concert with Spo0A. One such putatively interacting protein was isolated using Dynabeads M-280 Streptavidin. The respective gene was cloned and overexpressed in *E. coli*, allowing enrichment and partial characterization of its gene product. A Spo0A-mutant protein was constructed, which showed constitutive binding (without phosphorylation) to 0A-boxes and which will be used as a bait for identification of further interaction partners.

## STATIONARY PHASE, SPORULATION AND PATHOGENESIS OF *CLOSTRIDIUM DIFFICILE*.

A. L. Sonenshein, J. T. Nordman, and A. Villapakkam

Department of Molecular Biology and Microbiology, Tufts University School of Medicine, Boston, MA USA

Pathogenesis and sporulation of *Clostridium difficile* are connected in two significant ways: (i) The spore form of the bacterium is undoubtedly the major reservoir of infectious organisms, explaining its persistence and ubiquity in hospital settings and the ability of *C. difficile* to cause infection after patients are treated with high doses of antibiotics; (ii) the two major virulence factors, toxins A and B, are expressed under conditions that also induce sporulation (limitation of amino acids and exhaustion of rapidly metabolizable carbon sources). Although no gene required for sporulation is known to be essential for toxin synthesis, it is likely that the two processes reflect a common response to nutritional conditions. Previous work has shown that both the synthesis of toxin proteins and its response to environmental conditions depend on TxeR, a novel RNA polymerase sigma factor that is required for transcription of the toxin genes. It is synthesis of TxeR that is controlled by the environment. The response of the toxin genes may only be indirect. In our recent work, we have been investigating the roles in *txeR* and *tox* gene regulation of two regulatory proteins found ubiquitously in the low G+C family of gram-positive bacteria. In *Bacillus subtilis*, CodY is a GTP-activated repressor of many genes that are induced at the onset of stationary phase and sporulation. Interaction of CodY with DNA is also stimulated by the amino acids isoleucine and valine. We have cloned, expressed and purified *C. difficile* CodY and shown that it binds in vitro to the *txeR*, *toxA* and *toxB* regulatory regions, as well as to the promoter regions of known *B. subtilis* CodY target genes. Binding of *C. difficile* CodY is stimulated by GTP, isoleucine and valine. CcpA is a global regulator of carbon metabolism in gram-positive bacteria. We have cloned and expressed the *C. difficile* homolog of CcpA and are testing its interaction with the *txeR* and *tox* promoters.

## CHARACTERIZATION OF *spo0A* HOMOLOGUE IN ENTEROTOXIGENIC *CLOSTRIDIUM PERFRINGENS* TYPE A

M. R. Sarker<sup>1</sup>, M. Waters<sup>1</sup>, R. R. Grau<sup>2</sup> and I. Huang<sup>1</sup>.

<sup>1</sup>Department of Microbiology, Oregon State University, Corvallis, OR 97331 and <sup>2</sup>Department of Microbiology, Rosario University School of Biochemistry and Pharmacy, Rosario 2000, Argentina.

Spo0A is a member of the response regulators that control many of the changes in gene expression during the initiation of sporulation. Several studies have indicated that the *spo0A* homologue is present in different *Bacillus* and *Clostridium* species. Recent genome sequencing of *C. perfringens* strain 13 also identified a *spo0A* homologue with a frame-shift mutation in the ORF that generated two truncated Spo0A homologous proteins of 150 and 117 aa, respectively. In this study, to examine whether enterotoxin (CPE)-positive *C. perfringens* type A carry an intact *spo0A* homologue, both strands of an ~1.0-kb PCR amplified DNA insert containing the *spo0A* ORF from two chromosomal (NCTC8239 and SM101) and two plasmid (B11 and F4969) *cpe* isolates, were nucleotide sequenced. These analyses revealed that the *spo0A* ORF sequences present in all of the surveyed *cpe*-positive isolates differ by only one nucleotide (C instead of A at position 451 from ATG) from the *spo0A* sequence in strain 13. This single nucleotide change in *cpe*-positive isolates replaces the termination codon TAA found in strain 13 by TCA and forms an intact ORF encoding a Spo0A homologous protein of 276 aa. These results suggest that a functional *spo0A* homologue exists in *cpe*-positive isolates. In order to evaluate the contribution of Spo0A to the sporulation of *C. perfringens*, in our current study we constructed an isogenic *spo0A* knock-out mutant of SM101. Western blot analyses confirmed that the *spo0A* knock-out mutant failed to produce Spo0A, and this lack of Spo0A production could be complemented by a recombinant plasmid (pMRS122) carrying the wild-type *spo0A* gene. When the sporulation of wild type, mutant and complementing strains were compared, the wild type strain showed significant sporulation, but the *spo0A* mutant was unable to produce spores. However, full sporulation-phenotype could be restored by complementing the mutant with pMRS122, which confirms that the observed loss of sporulation for the *spo0A* knock-out mutant results from the specific inactivation of the *spo0A* gene and the resultant loss of Spo0A production. CPE-Western blotting demonstrated that the *spo0A* mutant failed to produce CPE, and this lack of CPE production could be complemented by pMRS122. The results from this study provide evidence that Spo0A production is necessary for the formation of spores and, thus, for the production of CPE in *cpe*-positive *C. perfringens* type A.

## EFFICIENT SPORULATION IN *CLOSTRIDIUM DIFFICILE* REQUIRES DISRUPTION OF THE $\sigma^K$ GENE

J. D. Haraldsen and A. L. Sonenshein

Tufts University School of Medicine, Boston, Massachusetts, 02111

*Clostridium difficile*, a major nosocomial pathogen, is associated with 10-25% of cases of antibiotic associated diarrhea (AAD) and almost all cases of pseudomembranous colitis (PMC). To test the postulated role of endospores as the infectious form of the organism, a better understanding of sporulation of *C. difficile* is needed. We therefore sought to identify homologs of genes known to regulate sporulation in *Bacillus subtilis*, e.g., the sporulation-specific  $\sigma$  factors of RNA polymerase.

During our analysis of *C. difficile*  $\sigma$  factors, we discovered that *sigK*, the gene for the latest-acting mother cell  $\sigma$ , is disrupted by a large prophage-like element termed *skin*<sup>Cd</sup> (*sigK* intervening element) in strain CD630. During sporulation the *skin*<sup>Cd</sup> element precisely excises from the gene, generating an intact *sigK* ORF. Until now *B. subtilis* has been the only spore-former shown to carry such an insertion. All other previously studied *Bacillus* and *Clostridium* species contain uninterrupted *sigK* genes. The *Bacillus* and *Clostridium* elements are divergent in sequence, inserted at different sites within the *sigK* gene, and are in opposite orientations.

The majority of *C. difficile* strains contain *skin*<sup>Cd</sup>, but we also identified two strains that lack this element. The absence of *skin*<sup>Cd</sup> correlates with a sporulation defect in these strains, unlike the case for *skin*-less mutants of *B. subtilis*. This defect may potentially be explained by an additional difference between the *B. subtilis* and *C. difficile* *sigK* genes. *B. subtilis*  $\sigma^K$  is not expressed in an active form, but rather as a pro- $\sigma$ . Activation of pro- $\sigma^K$  requires removal of 20 N-terminal amino acids. We have shown that in both *skin*<sup>Cd+</sup> and *skin*<sup>Cd-</sup> strains of *C. difficile* this pro sequence is entirely absent. Without this post-translational mechanism of regulation, the presence of *skin*<sup>Cd</sup> may be a required mechanism for regulation of  $\sigma^K$  activity. To test this hypothesis, we constructed by Tn916-mediated intergeneric conjugation a merodiploid strain that carries both the interrupted and uninterrupted versions of *sigK*. This strain was Spo<sup>-</sup>, showing that intact *sigK* is dominant and suggesting that premature activation of  $\sigma^K$  interferes with the sporulation pathway.

## PHOSPHATE LIMITATION IN *CLOSTRIDIUM ACETOBUTYLICUM*

H. Bahl, M. Mix, S. Oehmcke, T. Fiedler, K. Schwarz and R.-J. Fischer

Department of Biological Sciences, Division of Microbiology, University of Rostock, Rostock, Germany

Limitation of growth by the amount of phosphate supplied in the medium favours the onset of sporulation in clostridia. In addition, in *Clostridium acetobutylicum* phosphate limitation in combination with pH values below 5 results in a metabolic shift from acid to solvent formation. To elucidate the role of phosphate limitation within the complex regulatory network of sporulation and solventogenesis, we have started to analyse the phosphate regulon of *C. acetobutylicum*. The mRNA levels of two operons encoding a two-component regulatory system (tentatively designated *phoPR*) and an ABC-transport system (tentatively designated *pstSCABphoU*), respectively, were found to be up-regulated under phosphate limitation. Using the *lacZ* gene of *Thermoanaerobacterium thermosulfurigenes* EM1 as a reporter gene, a promoter which responds in *C. acetobutylicum* to low external phosphate concentrations could be located on a 165-bp fragment in front of *pstS*. Putative Pho boxes will be confirmed using the purified regulator PhoP of *C. acetobutylicum* for gel shift and footprint experiments. Interestingly, Northern blot analysis revealed that in a phosphate-limited chemostat at a pH-value of 4.5 (conditions optimal for solvent production) the level of the *pstS*-specific mRNA in *C. acetobutylicum* is decreased by a factor of eight and of the total *pst* operon by a factor of two. Under the assumption that no alternate high-affinity phosphate uptake system becomes active under these conditions, it might be that the impaired supply of cells with phosphate is crucial for the initiation of solvent formation (and sporulation). In agreement with our hypothesis we observed faster phosphate consumption in cultures of *C. acetobutylicum* at higher pH values.



11:00 AM-1:00 PM Session X: Host-Pathogen Interactions

ACTIVATION OF PLATELET gpIIbIIIa BY CLOSTRIDIUM PERFRINGENS PLC.

AE Bryant<sup>1,2</sup>, CR Bayer<sup>2</sup>, SM Hayes-Schroer<sup>2</sup>, DL Stevens<sup>1,3</sup>

<sup>1</sup>VA Medical Center, Boise, ID; <sup>2</sup>Univ of Idaho, Moscow, ID; <sup>3</sup>Univ of Washington, Seattle, WA.

*Clostridium perfringens* gas gangrene is characterized by rapid destruction of tissues and radical amputation remains the single best treatment. Our previous studies demonstrated that intramuscular injection of phospholipase C (PLC), the major virulence factor of *C. perfringens*, induced the formation of intravascular aggregates of platelets, leukocytes and fibrin that irreversibly occluded blood flow and contributed to tissue destruction. Further, aggregate formation was mediated by the platelet fibrinogen receptor, gpIIbIIIa. The present study investigated the intracellular signaling events leading to activation of gpIIbIIIa by PLC. Gel filtered platelets were analyzed by flow cytometry for expression of the activated conformation of gpIIbIIIa. PLC caused a time- and dose-dependent activation of surface expressed gpIIbIIIa and also mobilized gpIIbIIIa from internal stores, and blockade of activated gpIIbIIIa inhibited PLC-induced platelet aggregation. PLC-induced functional upregulation of gpIIbIIIa was the consequence of PLC's enzymatic activity and not receptor occupancy. Chelation of intracellular calcium strongly inhibited the activation of gpIIbIIIa by PLC, whereas inhibition of protein kinase C was without effect. Inhibitors of store-operated calcium entry also blocked PLC-induced activation of gpIIbIIIa and altered calcium mobilization in Fluo-4-loaded platelets. We conclude that PLC initiates an "inside-out" signaling cascade that begins with depletion of internal calcium stores, is sustained by an influx of calcium through store-sensitive channels and culminates in functional activation in gpIIbIIIa. These findings suggest that calcium channel blockade and therapeutic strategies targeting gpIIbIIIa, such as those currently used to treat acute myocardial infarction, may prevent vascular occlusion, maintain tissue viability, and provide an alternative to radical amputation for patients with clostridial gas gangrene.

## THE MOLECULAR MECHANISM OF *CLOSTRIDIUM PERFRINGENS* EPSILON-TOXIN CYTOTOXICITY.

A. Okabe<sup>1</sup>, S. Miyata<sup>1</sup>, E. Tamai<sup>1</sup>, J. Minami<sup>2</sup>, S. Shimamoto<sup>1</sup> and O. Matsushita<sup>1</sup>.

<sup>1</sup>Department of Microbiology, Kagawa, Japan. <sup>2</sup>Department of Medical Technology, Kagawa Prefectural College of Health Sciences, Kagawa, Japan.

*Clostridium perfringens* epsilon-toxin is the most potent clostridial toxin after botulinum and tetanus toxins, and is responsible for the pathogenesis of fatal enterotoxaemia of domestic animals caused by the organism. By whole-body autoradiography of epsilon-toxin injected mice, we have shown that it accumulates most prominently in the kidneys and fairly abundantly in the brain. Our results from animal model experiments have suggested that the accumulation in the kidneys is not responsible for the mice lethality, but instead contributes to the host defense. In our previous study, we showed that epsilon-toxin exhibits neurotoxicity in rats and mice, and suggested that it is most responsible for its lethal toxicity. The molecular mechanism underlying the neurotoxicity has been approached by examining whether or not the toxin forms a large complex in the synaptosomal membrane, as has been demonstrated for the MDCK cell membrane permeabilized by the toxin. We have shown the toxin assembles into heptamer in the synaptosomal membrane. The question of how minute quantities of epsilon-toxin assembles efficiently in the membrane has also been addressed. Our results indicate that the toxin binds preferentially to the detergent-insoluble microdomains of the synaptosomal membrane and forms heptamers therein. This may imply that epsilon-toxin utilizes the detergent-insoluble microdomains as a concentration device similarly to aerolysin.

COLLAGEN-BINDING DOMAIN OF CLOSTRIDIAL COLLAGENASE.  
GENETIC EVOLUTION-AND STRUCTURE-FUNCTION  
RELATIONSHIPS

O. Matsushita<sup>1</sup>, J. J. Wilson<sup>2</sup>, J. Sakon<sup>2</sup> and A. Okabe<sup>1</sup>

<sup>1</sup>Department of Microbiology, Kagawa Medical University, Kagawa, Japan.

<sup>2</sup>Department of Chemistry and Biochemistry, University of Arkansas,  
Fayetteville, AR USA.

Clostridial collagenases possess a collagen-binding domain(s) (CBD) at their C-termini in common. Their sequence analysis revealed that duplications of the domain were reiterated within a single gene and within this metallo-protease gene family. The crystal structure of the domain with an N-terminal domain linker from *Clostridium histolyticum* class I collagenase was determined at 1.00 Å resolution in the absence of calcium and at 1.65 Å resolution in the presence of calcium. In the absence of calcium, CBD reveals a beta-sheet sandwich fold with the linker adopting an alpha helix. The addition of calcium unwinds the linker and anchors it to the distal side of the sandwich as a new beta-strand. The conformational change of the linker upon calcium binding is confirmed by changes in the Stokes and hydrodynamic radii as measured by size exclusion chromatography and by dynamic light scattering with and without calcium. Furthermore, extensive mutagenesis of conserved surface residues and collagen-binding studies allow us to identify the protein's collagen-binding surface and propose likely collagen-protein binding models. Functionally important residues in CBD were well conserved throughout the collagenases from various species.

## DEATH PATHWAYS ACTIVATED IN CACO-2 CELLS BY CLOSTRIDIUM PERFRINGENS ENTEROTOXIN

G. Chakrabarti and B. A. McClane

Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, PA

*Clostridium perfringens* enterotoxin (CPE), a 35 kDa single polypeptide, causes symptoms of *C. perfringens* type A food poisoning, antibiotic associated diarrhea. It also induces cytotoxic effects in the CaCo-2 cell, which is often used as an *in vitro* enterocyte-like model. To identify the mammalian cell death pathway(s) mediating CPE-induced cell death, monolayer CaCo-2 cultures were treated with 1 µg/ml or 10 µg/ml of CPE. Both CPE doses induced morphologic damage and DNA cleavage in CaCo-2 cells. The oncosis inhibitor glycine, but not a broad-spectrum caspase inhibitor, transiently blocked both pathologic effects in CaCo-2 cells treated with the higher, but not the lower, CPE dose. Conversely, broad-spectrum caspase inhibitor (Z-VAD-FMK) and a caspase 3/7 inhibitor (DEVD-CHO) but not glycine or a caspase 1 inhibitor (YVAD-CHO) inhibited morphologic damage and DNA cleavage in monolayer CaCo-2 cells treated with the lower, but not the higher, CPE dose. Collectively, these results indicate lower CPE doses cause caspase 3/7-dependent apoptosis, while higher CPE doses induce oncosis. Apoptosis caused by the lower CPE dose was shown to proceed via a classical pathway involving mitochondrial membrane depolarization and cytochrome C release from mitochondria to cytosol, whereas oncosis caused by CPE does not involve mitochondrial membrane depolarization and cytochrome C release. Extracellular calcium was identified as a necessary component for both CPE-induced oncosis and apoptosis, and CPE was demonstrated to increase  $Ca^{2+}$  influx into CaCo-2 cells in a dose-dependent manner. These findings suggest that calcium influx levels influence whether oncosis or apoptosis death pathways become activated in CPE-treated CaCo-2 cells.

## PERSISTENCE OF *CLOSTRIDIUM PERFRINGENS* IN HOST TISSUES: LIFE INSIDE A MACROPHAGE?

S. B. Melville, M. E. Woodman, and D. K. O'Brien

Department of Biology, Virginia Polytechnic Institute and State University,  
Blacksburg, VA USA

*Clostridium perfringens* is the major cause of clostridial myonecrosis (gas gangrene). The bacteria is usually introduced into the tissues of the patient after trauma or surgery and can multiply and cause myonecrosis if the wound becomes ischemic. Once the bacteria begins to multiply, it can spread rapidly to healthy tissues, sometimes at the rate of several inches per hour. When myonecrosis is clinically evident serious complications usually follow, including extensive tissue debridement, amputation, or even death. Therefore, we have focused our research on the very earliest stages in a gangrene infection, where phagocytic cells have infiltrated a fresh wound and the bacteria have not yet begun to multiply. Both PMNs and macrophages have the capacity to phagocytose and kill bacteria, even under anaerobic conditions. The main question we have addressed is why these cells fail to kill *C. perfringens*. Surprisingly, we have found that murine macrophages are incapable of killing *C. perfringens* even under aerobic conditions. We have also demonstrated that *C. perfringens* can escape the phagosome inside macrophages and, apparently, persist in the cytoplasm. This phagosomal escape is mediated by the membrane active toxins PLC and PFO. In addition, PFO, but not PLC, has powerful cytotoxic activities against macrophages. In a murine model simulating conditions in the earliest stages of an infection (i.e., where the infectious dose is 1000-fold less than that needed to initiate a gangrene infection) both PFO and PLC are required for persistence of *C. perfringens* in mouse muscle tissue. In contrast, we have shown that human PMNs and monocytes are capable of killing *C. perfringens* in vitro assays, if complement is present. These facts lead us to propose a model for how *C. perfringens* can persist in murine tissues: Most of the bacteria are phagocytosed and killed by PMNs but some of the bacteria are phagocytosed by macrophages. Inside the macrophage, the bacteria can escape into the cytoplasm and reside there for a significant period of time (e.g., several days), protected from PMN-mediated killing. If the wound becomes ischemic, the bacteria can grow rapidly outside the cells and initiate a gangrene infection. This model may explain some cases of delayed onset gangrene infections, which occur some time after the initial trauma.

## S-LAYER AND RELATED SURFACE PROTEINS OF *CLOSTRIDIUM DIFFICILE*: MOLECULAR AND FUNCTIONAL PROPERTIES AND POSSIBLE ROLES IN VIRULENCE

Neil Fairweather and Emanuela Calabi

Department of Biological Sciences, Centre for Molecular Microbiology and Infection, Imperial College, London, UK

*Clostridium difficile* is the aetiological agent of antibiotic-associated diarrhoea (AAD), a potentially serious condition frequently affecting elderly hospitalised patients. Whilst tissue damage is primarily induced by toxins A and B, the mechanism of gut colonisation, and particularly the role of bacterial adherence to the mucosa, remains to be characterised.

We have characterised in detail the molecular biology and functions of the Surface Layer Proteins (SLPs), the predominant cell wall components in *C. difficile*. We have identified a family of related proteins within the genome sequence of strain 630, suggesting multiple surface localised proteins. Bioinformatics analysis suggests that some members of this family may play roles in adherence or colonisation of the host.

We have studied the properties of the SLPs in detail at the genetic and molecular level as well as examining potential functions *in vivo*. The SLPs appear to mediate binding to eukaryotic cells as shown by ELISA and FACS analysis. Antibodies to the high MW SLP inhibited adherence, suggesting a role for this protein in colonisation of the bacteria. Immunohistochemical analysis on human gastrointestinal tissue sections revealed strong binding of the high MW SLP both to the surface epithelium lining the digestive cavities and to the lamina propria. SLPs were shown to bind collagen I, thrombospondin and vitronectin, but not to collagen IV, fibronectin or laminin. These results raise the possibility that the SLPs play a role both in the initial colonisation of the gut by *C. difficile* and possibly in the subsequent inflammatory reaction.

**IMMUNIZATION WITH C-DOMAIN OF ALPHA TOXIN PREVENTS  
LETHAL INFECTION, LOCALIZES TISSUE INJURY AND PROMOTES  
HOST RESPONSE TO CHALLENGE WITH VIABLE *CLOSTRIDIUM  
PERFRINGENS***

**DL Stevens<sup>1,2</sup>, RW Titball<sup>3</sup>, M Jepson<sup>3</sup>, C Bayer<sup>4</sup>, S Schroer<sup>4</sup>, AE Bryant<sup>1,4</sup>**

<sup>1</sup>VA Med Ctr, Boise, ID; <sup>2</sup>Univ of Washington, Seattle, WA; <sup>3</sup>CBD Sector  
DERA Porton Down, Salisbury Wiltshire, UK; <sup>4</sup>Univ of Idaho, Moscow, ID.

*Clostridium perfringens* gas gangrene is a life threatening deep soft tissue infection characterized by rapid destruction of tissue and an absence of granulocytic influx. Previous studies have implicated phospholipase C (PLC; alpha toxin) as the virulence factor most responsible for these pathologies. In the current study, Swiss Webster mice were actively immunized against the C-terminal domain of alpha toxin (Cpa247-370). A sham-immunized group of mice served as negative controls. The development of anti-PLC antibody by animals immunized against the C-domain was verified by western blotting. Mice (22/group) were then challenged with either  $3.2 \times 10^8$  or  $3.2 \times 10^9$  washed, log phase *C. perfringens* (ATCC 13124). Animals (10/group) were closely observed for signs and symptoms of local and systemic infection and mortalities recorded. The remaining mice (2/time point) were sacrificed at 0, 0.5, 1.5, 2.5, 4.5 and 6.5 hr post challenge. Tissue from the site of inoculation was harvested, fixed, sectioned stained with hematoxylin/eosin and examined for evidence of myonecrosis and granulocytic influx.

In the sham-immunized groups, mortalities of 100% were observed within 9-15 hr. In contrast, mortality was 10% and 20% at 18 hr post infection in the C-domain-immunized mice challenged with  $3.2 \times 10^8$  and  $3.2 \times 10^9$ , respectively. No significant difference in localized swelling was observed between controls and C-domain-immunized animals. In contrast, 100% of sham-immunized animals in the high inoculum group developed blackened ischemic feet, whereas only a transient darkening of the foot was observed in 20% of those immunized against the PLC C-domain. Histopathology demonstrated reduced muscle necrosis and enhanced influx of granulocytes in C-domain-immunized mice compared to sham-immunized controls. We conclude that immunization with the PLC C-domain reduces the morbidity and mortality of *C. perfringens* gas gangrene by maintaining tissue perfusion, reducing localized tissue destruction and improving leukocyte influx to the site of infection.